Elucidating protein-lipid interactions in wheat gluten using acetic acid

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

Thu Hoa McCann (nee Vu)
B.Sc. Food Sci Tech (Hons) RMIT University

School of Applied Sciences
Science, Engineering and Technology Portfolio
RMIT University

December 2007

Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Thu McCann

December 2007

Acknowledgments

Firstly, I would like to express my special thanks to my supervisors Dr Li Day, Dr Colin Wrigley, Dr Ian Batey and Dr Darryl Small for their continual support, guidance and enthusiasm throughout my research and during preparation of this thesis.

I gratefully acknowledge the Value Added Wheat Co-operative Research Centre (VAWCRC), North Ryde and their industry partner, Manildra Milling Corporation for financially supporting this project, particularly through the award of a scholarship. I also received assistance to attend the Gluten Workshop and AACC International conference in San Francisco during September 2006 through the award of a scholarship by AACCI Student Travel Award Committee.

I am grateful to Food Science Australia, Werribee, for granting me a period of study leave for three years so that I could undertake this project. In addition, the use of facilities at Food Science Australia, Werribee, throughout this project is acknowledged. I would like to thank all my friends from the Microbiology, Chemistry and Bio-processing groups for their warm friendship, support and encouragement during the last three years.

I would like to thank the RMIT University for giving me the opportunity to conduct this research. I am also grateful for the support and warm friendship from post-graduate students within Applied Chemistry, in the School of Applied Sciences, RMIT University.

A number of other people helped me during my research and I would particularly like to thank Dr Peter Fagan from Food Science Australia, Werribee for technical support and valuable discussions on the analysis of lipids by HPLC, Dr Daniel Skylas, Australian Proteome Analysis Facility (APAF), North Ryde, who provided training on setting up and running the two dimensional gel electrophoresis system and Dr Cheryl Taylor, Food Science Australia, Werribee for showing me how to operate the P/ACE System for protein analysis by capillary electrophoresis.

I also thank Mrs Gail Digregorio for technical assistance in determining protein content of my samples using the Leco system and lipid content using the hydrolysis method. I acknowledge the analysis of amino acid composition of gluten fractions carried out by the Australian Proteome Analysis Facility (APAF), North Ryde and the helpful discussions from Dr Chakra Wijesundera, Food Science Australia, Werribee, on the aspect of lipid properties and Dr Roderick Williams, Food Science Australia, Werribee in the area of protein fractionation.

I would like to thank my Mother, Father, all my sisters and brothers and my parents-in-law, Cliff and Angela for their love, encouragement and support throughout my studies.

Finally, I would like to specially thank my husband, Karl for his love, patience and constant support and encouragement as well as useful discussions on technical issues of protein fractionation and analysis throughout my studies.

Publications and presentations

Journal article

Day, L., Vu, T., 2004. Lipids in wheat flour and breadmaking. Lipid Technology 16, 275-279.

Conference Proceedings

Vu, T., Day, L., Small, D.M., 2004. Protein-lipid interactions in gluten. Proceedings of The 12th annual RACI Analytical and Environmental Research & Development topics 2004, 5-8 Dec, Melbourne.

Vu, T., Small, D.M., Day, L., 2005 Combining a sequential extraction method with an acetic acid fractionation technique in studying lipid-protein interactions in gluten. Proceedings ICC-Jubilee Conference "Cereal – The future challenge", 3-6 July, Vienna.

McCann, T., Small, D.M., Day, L., 2005. Study of lipid-protein interactions in gluten using a 3-step solvent extraction and acetic acid fractionation techniques. In: Blanchard, C.L., Truong, H., Allen, H.M., Blakeney, A.B., and O'Brien, L. (Eds.), Cereals 2005. Royal Aust. Chem. Instit., Melbourne, pp. 273-276. ISBN: 1-876892-12-5.

McCann, T.H., Small, D.M., Batey, I.L., Wrigley, C.W., Day, L. 2006. Probing protein-lipid interactions in gluten – An acetic acid fractionation approach. In: Lookhart, G.L. and Ng, P.K.W. (Eds.), Gluten Proteins 2006. AACC International, St. Paul, Minnesota, pp. 178-182. ISBN: 978-1-891127-57-1.

McCann, T., Dela Cruz, C., Day, L. Separation of vital wheat gluten to gliadin-rich and glutenin-rich fractions. 2006. Proceedings of World Grains Summit: Foods and Beverages. AACC International and The Master Brewers Association of the Americas, 17-20 Sept, San Francisco, pp. 171.

McCann, T.H., Small, D.M., Batey, I.L., Wrigley, C.W., Day, L. 2007. Protein and lipid distribution in the gluten matrix altered by acetic-acid treatment. In: Panozzo, J.F. and Black, C.K. (Eds), Cereal 2007 – Proceedings of 57th Australian Cereal Chemistry Conference. Royal Australian Chemical Institute, Melbourne, pp. 159-162. ISBN: 1-876892-16-3.

Abstract

Wheat gluten continues to be used widely as an ingredient in many food products, particularly in the bakery industry, due to its ability to form a gluten matrix. Lipid, a relatively minor component of gluten, has been found to be associated with proteins in this structure and might partially contribute to the high extensibility and elasticity of gluten. Currently, knowledge of protein-lipid interactions in the gluten complex is limited; therefore, a study of this area could provide an enhanced understanding on the application of gluten in food products. Accordingly, this research firstly aims to investigate the protein-lipid interactions on the basis of alterations in lipid and protein distribution following various treatments and fractionation using acetic acid. Secondly, the presence of specific protein-lipid interactions in gluten is further studied through various analyses of the protein composition of the gluten fractions.

Initially, the free and bound lipid components from flour and gluten were separately extracted using a three-step solvent extraction with petroleum ether, chloroform and ethanol. All lipid classes in these extracts were determined using an HPLC method. A comparison of the composition of free and bound lipid from flour and gluten indicated that most of the non-polar lipids originally found in the flour were retained in the gluten with an increasing level of the bound form. A small proportion of the glycolipids and phospholipids from flour were recovered in the gluten but all were present in the bound form. This demonstrates that non-polar lipids could be entrapped within the gluten structure or, alternatively, may become closely associated with at least some of the protein components during gluten preparation.

In the investigation of protein-lipid interactions using acetic acid, three distinct approaches have been carried out. The first was to treat gluten with two different concentrations of acetic acid (0.01 and 0.1 M). Following these treatments, non-polar lipid became more readily extractable with solvents of low polarity whilst the level of bound glycolipids and phospholipids were not affected. These results indicate the disruption of the hydrogen bonds within the gluten matrix, allowing some lipids to be accessible to extraction with the non-polar solvent. In addition, glycolipids and

phospholipids might associate with gluten protein in specific ways that were not affected under the acidic conditions.

In the second approach, gluten protein was fractionated into supernatant and pellet using various concentrations of acetic acid (0.01, 0.05 and 0.1 M). Gliadins were found to be the majority of the proteins solubilised at the lower acetic acid concentration (0.01 M) whilst glutenins were more soluble at the high concentrations of acetic acid (0.05 and 0.1 M). The higher levels of free non-polar lipids in the pellet of the lower acetic acid (0.01 M) fractionation indicate that the non-polar lipid is probably associated with gluten proteins through hydrophobic bonds. Results also showed that non-polar lipid and glycolipid are probably associated with glutenin as a complex, which becomes more soluble at the higher acetic acid concentrations (0.05 and 0.1 M). Phospholipids were preferentially extracted with the gliadins, indicating the interaction of phospholipids with these proteins.

The third approach utilised sequential fractionation with acetic acid at a low concentration (0.01 M) followed by a higher concentration (0.1 M). The fraction soluble in the more dilute acetic acid was predominantly gliadins, while that soluble at the high higher concentration contained both gliadins and glutenin. Most free lipid was present in the 0.1 M acetic acid soluble fraction, with non-polar lipid being the major component. The bound lipid of the 0.1 M acetic acid soluble and acetic acid insoluble fraction contained the highest proportion of glycolipid, a medium amount of non-polar lipid and the lowest proportion of phospholipid. On the other hand, the 0.01 M acetic acid soluble fraction had the highest proportion of phospholipid and the lowest proportions of non-polar lipid and glycolipid within the bound lipid.

Various approaches were applied to the identification of interactions between specific proteins and particular lipid classes, particularly the presence of lipoproteins in the gluten fractions. The changes in the amounts of the protein and lipid components in the gluten fractions, demonstrate that the low molecular weight gluten subunits (LMW-GS) and gliadins at specific molecular weights may associate with phospholipids or glycolypids. The results provide evidence that different types of lipid-binding protein are present in the gluten fractions and that there are specific interactions between lipid binding proteins and phospholipid components within the fractions.

On the basis of the pattern of proteins, in conjunction with the distribution of free and bound lipid in the gluten fractions it is concluded that the entrapment of lipid in gluten occurs only for the non-polar lipids, whilst polar lipids interact with proteins through specific bonding mechanisms. The data also indicates that glycolipid is probably associated with glutenin through hydrogen bonds and hydrophobic interactions while the phospholipids preferentially interact with gliadins. These findings have been used as the basis for the development of a new model of the interactions between proteins and lipids in the gluten matrix.

Table of contents

List of tables

List of figures

Abbreviations

2DE	Two-dimensional electrophoresis
AACC	American Association of Cereal Chemists
A-PAGE	Aluminium lactate/lactic acid-polyacrylamide gel electrophoresis
AUC	0.1 M Acetic acid, 3 M urea, 0.01 M cetyltrimethylammonium bromide
CD	Circular dichroism
CE	Capillary electrophoresis
CM protein	Chloroform/methanol soluble protein
DAG	Diacylglycerols
DGDG	Digalactosyldiglyceride
DMPG	1,2 dimyristoylphosphatididyl glycerol
ELSD	Evaporative light scattering detector
FAME	Fatty acid methyl ester
FFF	Flow field-flow
FZCE	Free-zone capillary electrophoresis
g	Acceleration due to gravity
GLC	Gas liquid chromatography
HMW	High molecular weight
HMW-GS	High molecular weight glutenin subunits
HPCE	High performance capillary electrophoresis
HPLC	High-performance liquid chromatography
HPMC	Hydroxypropylmethylcellulose
IEF	Isoelectrofocusing
JCS	Journal of Cereal Science
LMPC	lyso-myristoylphosphatidylcholine
LMW	Low molecular weight
LMW-GS	Low molecular weight glutenin subunits
LPC	Lysophosphatidylcholine
LTP	Lipid transfer proteins
MAG	Monoacylglycerols
MALDI-TOF MS	Matrix-assisted laser desorption/ionisation time of flight mass spectrometry

MGDG	Monogalactosyldiglyceride
MW	Molecular weight
NEPHGE	Nonequilibrium pH gradient electrophoresis
nsLTP	Non-specific lipid transfer proteins
NSW	New South Wales
PAGE	Polyacrylamide gel electrophoresis
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PIN	Puroindolines
PIN-a	Puroindoline-a
PIN-b	Puroindoline-b
PS	Phosphatidylserine
Qld	Queensland
R^2	Coefficient of determination for a regression curve or line
RP-HPLC	Reverse phase-high performance liquid chromatography
rpm	Revolutions per minute
SA	South Australia
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
SE-HPLC	Size exclusion-high performance liquid chromatography
std	Protein standard
stdev	Standard deviation
TAG	Triacylglycerols
Tas	Tasmania
TFA	Trifluoroacetic acid
TLC	Thin-layer chromatography
TX114	Triton X114
UK	United Kingdom
US	United States of America
UV	Ultraviolet
Vic	Victoria
WA	Western Australia
W-FABP	Fatty-acid-binding protein from wheat kernel

WSB	*n*-butanol saturated with water

Explanatory notes

The purpose of these notes is to briefly describe the approaches adopted during the preparation of this thesis. They relate to the nomenclature of lipid classes and other chemical compounds, units of measurement, the expression of analytical results, as well as the referencing of literature sources:

1. In the naming of chemicals including lipid classes, the most recent recommendations of the International Union of Pure and Applied Chemistry have been followed (see Chapter 3).

2. The structures of some glycolipids and phospholipids presented in Chapter 3 have been drawn using CS ChemDraw Ultra® software (version 10.0) supplied by CambridgeSoft Corporation, Cambridge MA.

3. Generally experimental data is presented on a fresh weight (or as is) basis unless otherwise clearly specified.

4. It is emphasised that throughout this thesis, gluten samples were prepared from flour samples milled from known cultivars of wheat. The cultivars used were Lang (hard wheat) and Rosella (soft wheat). In all cases the gluten prepared was dried by freeze-drying and stored at −20°C prior to further analysis and fractionation. The gluten samples were defatted immediately prior to commencement of the fractionation studies. Therefore the control and treatment samples described at each stage of the thesis relate to the defatted gluten samples.

5. It is also noted that the defatting procedure was specifically designed to remove those lipid molecules which were not strongly interacting with other components. Therefore, the comparisons are made directly between the results obtained for the control gluten samples which had already been defatted and the fractions resulting from the various treatments.

6. In the citation and listing of references and information sources, the current recommendations of Elsevier Publishing for the Journal of Cereal Science (JCS. 2006) have been applied throughout (see page 237).

Chapter 1

Introduction

Wheat gluten is used around the world as a food ingredient, particularly in bakery products. It is produced commercially from wheat flour through four stages of processing which involve dough mixing, followed by resting, starch removal by washing and finally drying. Gluten has the potential to enhance the sensory attributes of a wide range of food products, due to its ability to form a gluten matrix with unique functional properties of extensibility and elasticity. Lipid, occurring at a moderate proportion in gluten, is believed to be involved in this structure and might contribute to the special properties of gluten.

Lipid in flour has been shown to impact on the quality of bread and other bakery products. The effects of different lipid components vary, with non-polar lipids having a negative affect on loaf volume, whilst polar lipids are beneficial for the volume and texture of bread. Polar lipids have also been found to influence cake volume and texture of biscuits. There is evidence that the effects of lipid on the quality of bakery products can be attributed to interactions occurring between lipid and protein.

Although derived from flour, gluten is quite different in chemical composition. Flour has relatively low proportions of lipid and protein, while gluten contains much higher levels of both lipid and protein. Furthermore, in comparison with flour, the lipid in gluten is less readily extractable with non-polar solvents. It is difficult to remove all the lipid from gluten without disrupting its structure and therefore functionality. These observations on lipid in gluten may also reflect the interaction of lipid and protein components.

Various studies have sought to elucidate these interactions in dough and bread using a range of chemical approaches and physical techniques. The specific interactions between lipid and protein identified from chemical approaches are highly dependent on the fractionation method employed. Recently, lipid-binding proteins have been isolated from wheat flour and their interactions with purified lipids studied at a molecular level.

On the other hand, the results from chemical studies appear to be inconsistent with those obtained with physical analyses. The latter demonstrate that lipid and protein do not interact in dough and gluten through the formation of specific bonds at a molecular level, but, rather that lipid is retained within the gluten matrix in a non-specific way. The relative significance of the various interactions and their impact on gluten functionality remains unresolved.

Accordingly, the development of this research project was based upon the hypothesis that the low extractability of lipid in gluten can be partially attributed to the entrapment of lipid within the gluten structure, whilst interactions of protein and lipid at the molecular level also occur. Therefore the broad aim of this project has been to investigate these interactions by analysing changes in the distribution of specific lipid and protein components following the extraction and fractionation of gluten.

Chapter 2

Background and literature review: The production and application of wheat gluten

The purpose of this chapter is to provide background and literature review on flour and wheat gluten production. The areas covered are the Australian wheat industry, flour milling, as well as the production of wheat gluten and its significance in food and non-food applications.

2.1 The Australian wheat industry

Wheat is one of the most important commodities produced by the Australian agricultural industry. The wheat grown in Australia is predominantly of white-grained varieties and these are preferred due to the higher rate of flour extraction which can be achieved whilst providing good flour colour. The two species of wheat commonly grown in Australia are *Triticum aestivum* and *T durum*. The former is known as common milling wheat or bread wheat and is used to produce a wide range of breads, Asian noodles and other bakery products, while the latter is primarily used for making pasta and couscous.

Wheat is grown widely across Australia where there are suitable conditions of soil type, soil fertility, topography and climate, particularly rainfall. This encompasses large areas of mainland Australia in an area west of the Great Dividing Range, known as the wheat belt, which stretches from Central Queensland through New South Wales and Victoria and on to South Australia, continuing into the south west of Western Australia as well as a small area in Tasmania. In 2003–04, almost 30,000 farmers in Australia grew wheat, using half of the agricultural land available for cropping and produced 26 million tonnes in total (Australian Bureau of Statistics, 2006). Yield rates vary from state to state, due to the variation in soil fertility and rainfall conditions in different regions. Wheat production of Australia in 2003–04 is summarised in Table 2.1, showing that Western Australia had the most area under wheat and the largest crop, NSW had the

greatest number of farms growing wheat and Tasmania produced the highest average yield.

Table 2.1 Australian wheat production in 2003–2004

	Number of farms	Area under wheat 1000 ha	Total production 1000 tonnes	Yield Tonnes/ha
NSW	10,859	3,983	7,288	1.8
Vic	5,743	1,409	3,145	2.2
Qld	2,035	790	1,110	1.4
SA	5,542	1,960	3,490	1.8
WA	5,053	4,917	11,070	2.3
Tas	289	8	26	3.4
Australia	29,524	13,067	26,132	2.0

Source: (Australian Bureau of Statistics, 2006)

Most of the wheat grown in Australia is exported with total exports representing approximately 15% of the world wheat trade annually whilst Australia typically produces only 3% of world wheat output. Australia's main wheat export markets are concentrated in Asia and the Middle East. During the past three years, Indonesia, Egypt, Iraq and Japan have been the leading importers (Table 2.2). Australia's wheat trade with China has been significant over a long period, but fluctuates (Table 2.2), depending on variations in regional production within China.

2.2 Flour processing

The production of flour from wheat grain through the milling process has developed over a long period of time and is now a relatively complex process as a result of the introduction of sophisticated technological advances (Cornell and Hoveling, 1998a). Prior to milling, wheat is cleaned and conditioned. Firstly, various sieves and air currents are utilised in order to remove foreign material that affect the quality of the resultant flour. Conditioning is carried out to avoid the powdering of the outer, bran layers of the wheat grain, to facilitate the separation of the endosperm from the bran, as well as mellowing the endosperm and minimising the level of starch damage. This is

achieved by adding sufficient water to increase the moisture content to a level appropriate for the particular wheat. This may be within the range of 13.5 to 16.5 % with lower levels for softer wheats and higher being applied to hard grained wheats (Posner and Hibbs, 1997).

Table 2.2 Destinations of Australian wheat exports

Importing country	2002–03 1000 tonnes	2003–04 1000 tonnes	2004–05 1000 tonnes
Iran	1,064	-	-
Egypt	602	2,534	752
Japan	1,123	1,239	1,171
South Korea	1,014	1,065	1,208
Iraq	1,037	1,111	1,550
China	38	750	1,883
Indonesia	1,578	2,492	2,501
Other	4,145	5,394	6,191
Total	10,601	14,585	15,256

Source: (Australian Bureau of Statistics, 2006)

The milling process involves a series of rollers and then shaker sieves to facilitate the separation of flour from the other parts of the grain including the bran and germ. The roll systems can be divided into two types: break and reduction rolls. The break system consists of sets of fluted rolls which function to open the grain up and release the endosperm as large particles. The successive sets of break rolls are set with gradually decreasing roll gaps and the sets of sieves are correspondingly finer (Posner and Hibbs, 1997; Cornell and Hoveling, 1998a).

Following the removal of bran flakes by sieving, the coarse endosperm particles produced within the break system are transferred to the reduction system. In this, the sets of rolls are smooth so that the endosperm material is reduced in size. The sieving system is used to effectively separate different types of flour and to remove fine bran fragments. The milling process provides a series of flour streams as well as bran and

other materials generally referred to as “offals”. The flour streams are mixed according to the requirements of the flour processor so that the flour yields are typically varied from approximately 72% for white flour, ranging up to 100% for wholemeal flour. Lower extraction rates are employed for some specific end-uses and typically the germ is excluded in order to minimise rancidity. A typical milled flour contains a large amount of starch (72–78%) and smaller amounts of protein (12–16%) and lipid (1–2%) (Mugford and Batey, 1989). Although proteins and lipids are not the predominant component of flour, they have an important impact in baking properties; these issues are reviewed in Chapters 3 to 5.

2.3 Wheat gluten production

Gluten is a sticky and water-insoluble fraction obtained from wheat flour. It has been reported that gluten was initially prepared from wheat flour almost 300 years ago with a simple water-washing experiment (Bailey, 1941). Over time, this washing procedure has evolved as an industrial process with the primary objective of starch production. For a long time, gluten was regarded as a by-product, as the material remaining after the starch-washing process. More recently, wheat gluten has become a valued ingredient having a variety of applications.

The basic procedure for gluten production involves mixing flour and water to form dough, resting the dough in water, washing the starch and drying the gluten. On a commercial scale, gluten is produced using either the Martin or the Batter process and these are suited to continuous and batch operation, respectively (Batey, 2004). The Martin process can be considered as the oldest for wheat gluten processing and still is the most widely used method. The Martin method is described in Figure 2.1 although it is noted that many variations have been applied to this basic approach to gluten production. The hydrated dough is washed with sufficient water to remove starch while it is being passed through various devices including ribbon blenders, rotating drums, twin-screw troughs and agitator vessels, and a tumbling cylindrical agitator with the action of kneading or rolling (Ponte et al., 2000). The Martin process is particularly well suited for low protein (7–10%) flours; however, it is does use a relatively large amount of water.

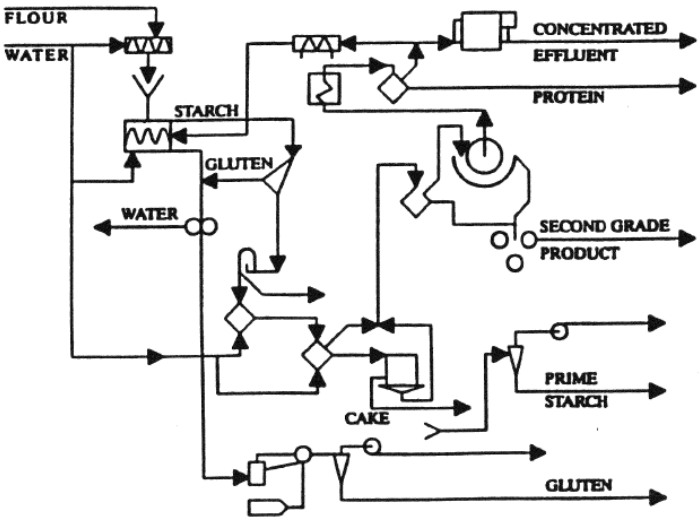


Figure 2.1 The Martin process for starch and gluten separation
Source: (Grace, 1988) from (Ponte et al., 2000)

In the Batter process for starch-gluten separation, dough with a solids content of 48–55% is stirred slowly in a water tank for several hours to separate the starch from the protein. The gluten protein is collected using a fine sieve that allows the starch to pass through whilst the protein is recovered as many curds remaining on the screen. This process can be repeated several times until all of the starch is washed out. A simplified representation of the Batter process is shown in Figure 2.2.

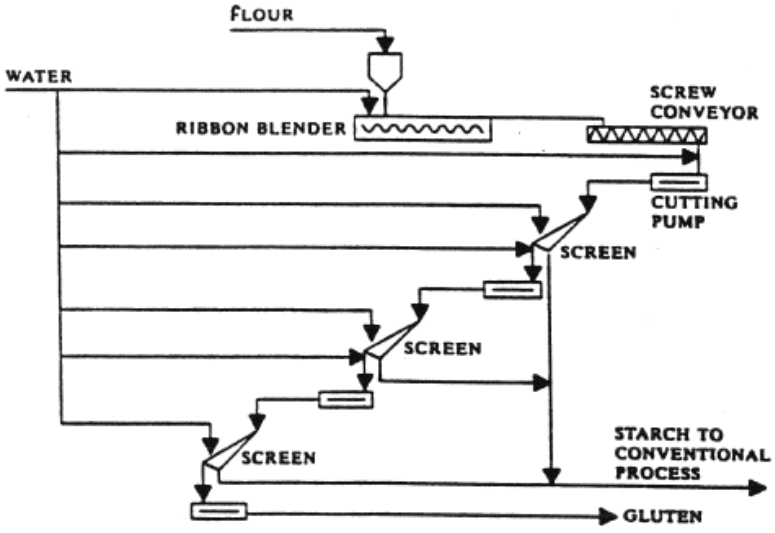


Figure 2.2 The Batter process for the separation of starch and gluten
Source: (Grace, 1988) from (Ponte et al., 2000)

The wet gluten produced using both of these processes can be stored in this form for only a few hours as it is subject to rapid deterioration. This is due to the activity of proteolytic enzymes occurring in the flour and also from microbial growth, and therefore, gluten is generally dried. The necessity for drying raises the issue of the impact of heat on the gluten proteins. There is a requirement for any drying procedure to avoid the possibility of causing denaturation or impacting on gluten functionality and its ability to retain the rheological characteristics for application in a variety of end uses. The term vital wheat gluten is commonly used to refer to preparations, which retain the important functional attributes of the gluten proteins.

In relation to the drying of wet gluten, for many years this has been achieved by processing under vacuum and low temperature, giving a product having a high vitality, pale colour and bland flavour; however, this process is relatively costly (Cornell and Hoveling, 1998c). Another way of drying gluten involves dispersion in either aqueous ammonia or acetic acid solution followed by spray-drying. This maintains the physical properties of gluten, although cost and environmental concerns limit this application. Currently, a ring drier is used to produce dry vital gluten in large-scale production. This technique effectively avoids the loss of visco-elastic properties that might occur due to the heat sensitivity of wet gluten. As wheat gluten is less prone to damage at lower moisture contents, in the ring drying process, wet gluten is firstly mixed with sufficient dry gluten to achieve a moisture of approximately 20%, after which it is comminuted and subjected to flash drying (Batey, 2004).

Commercial dry gluten typically contains approximately 75% protein and small amounts of lipid (4–8%), starch, residual moisture and fibre (Ponte et al., 2000). Most of the lipid originally present in the flour becomes associated with the protein during the gluten production process (Chung, 1986). This association between protein and lipid has an affect on the rheological properties of the gluten, as well as the lipid extractability and the practical application of the gluten product. The composition of the lipid and protein fractions in gluten will be discussed in Chapters 3 and 4, respectively. Further discussion on the interactions of protein and lipid in flour and gluten is the subject of Chapter 5.

2.4 Overview of wheat gluten industry

In Australia, vital dry gluten was first commercially produced at Fielder near Tamworth, NSW in 1933 and this formed the beginning of the starch and gluten industry. Total production was low initially; with 300 tonnes of flour used in 1935, 1,800 in 1940, increasing to over 20,000 by 1954 (Farrer, 1988). The industry has continued to develop so that today, Australia is one of the largest global suppliers of vital wheat gluten, producing more than 60,000 tonnes annually and representing 36.9% of gluten imports to the US market (Figure 2.3).

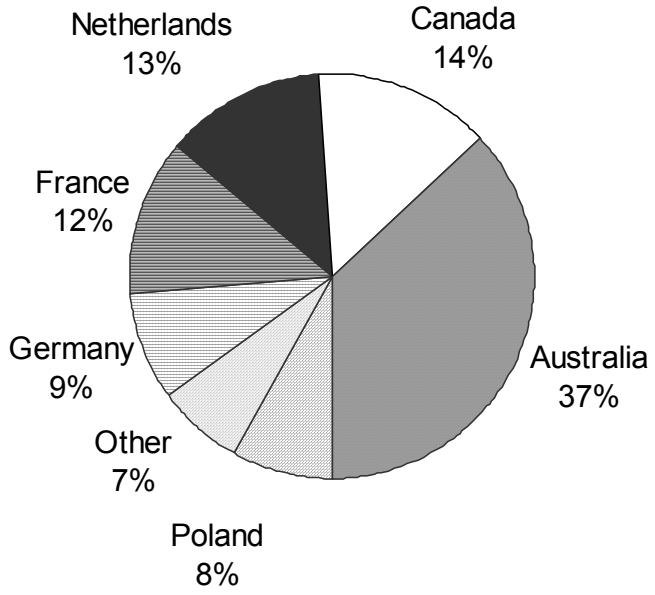


Figure 2.3 Sources of wheat gluten imported by the US from 1996 to 2004
Note: Values are averages expressed as a percentage of total imports
Source: (Boland et al., 2005)

Globally, the growth of the population, and the increasing consumer preference for "healthy" grain-based products, has been driving the demand for vital wheat gluten. During the past ten years, worldwide production of vital wheat gluten has increased. In the US, the gluten-plant capacity grew from approximately 73,000 tonnes in 1993 to 124,000 tonnes in 1997, with a 28% increase in 1994 and a 22% increase in 1995

(Holcomb, 2000). European production reached more than 300,000 tonnes per year in 2000 and has continued to increase. Recently, Cargill, one of the largest food-ingredient companies in the world, has announced plans to open the first vital wheat gluten production facility in Russia with the investment of US$60 million (Cargill, 2006).

2.5 Applications of gluten in food and non-food products

Traditionally, the most important application of gluten has been in the flour fortification and bakery industries (Table 2.3). Due to its unique visco-elastic properties, gluten can be used to fortify a flour of low protein content. In this case, addition of gluten can achieve the required protein content in flour, therefore, improving the strength of dough, mixing tolerance and the texture of bread (Cornell and Hoveling, 1998c). Gluten fortification of natural low protein bread flour has offered an attractive alternative to the use of expensive high-protein wheat while still satisfying functional performance requirements (Day et al., 2006). High usage of gluten for milling occurs in Europe, reflecting the need to strengthen the relatively weak bread flours generally produced in that area (Ponte et al., 2000). Australia, and particularly North America, use wheat gluten primarily in baking (Table 2.3). Gluten supplementation in the bakery industry is used to control the strength of dough and achieve the required performance for specific products. Increasing the protein content in dough by adding gluten can produce stronger dough, which has greater mixing tolerance for the production of refrigerated or frozen doughs. Gluten is also used to prepare high-protein bakery products considered as "healthy" foods for diabetics or to combat obesity. In addition to the varied milling and baking applications of gluten, considerable quantities are used for pet food and aquaculture feed in many areas of the world. A large amount is utilised in the manufacture of breakfast cereals in Australia as well as in the production of noodles and sausages in Japan (Table 2.3).

Among the properties of gluten are an excellent water absorption and fat-binding capacities; therefore, it has been used as a binding agent in meat, fish and poultry products including processed as well as restructured meats (Day et al., 2006). In meat systems, emulsions containing gluten, soy or milk protein result in the best consistency and organoleptic quality of the final product; however, the thermal stability appears to

be relatively poor for emulsions containing gluten (Örnebro et al., 2000). The wide range of applications for gluten in breakfast cereals, extruded snacks and fruit bars provides the nutritional value, flavour, and particularly texture that are desired for these products. Furthermore, gluten is also used for the purpose of meat replacement in vegetarian foods, artificial forms of seafood and crab in Japanese cuisines as well as production of monosodium glutamate and soy sauce. Recently, other applications of gluten have been developed in the food industry as gluten acts as a replacement for gelatine in chewy candy and fruit chews (Van and Schueren, 2002) and as a clarifying agent of must and white wines (Marchal et al., 2002).

Table 2.3 Wheat gluten consumption by end use

End use	Australia	North America	Europe	Japan
Baking	54	83	17	30
Flour fortification	9	1	66	-
Meats	9	1	-	25
Breakfast cereals	12	1	-	-
Noodles	-	-	-	10
Sausages	-	-	-	12
Pet foods	13	12	13	-
Others	3	0.6	4	23

Note: Values are expressed as percentage of total usage for the region
Source: (Day et al., 2006)

As the price of gluten is comparatively low, modified gluten has recently been attempted as a replacement of casein and soy isolates in food products. The modification is based on increasing the solubility of gluten that can be achieved by either chemical or enzymic modification (Batey, 2004). Such modification might enhance particular physical functional properties of gluten that might be suited to certain food systems. However, functional properties of proteins also depend on protein interactions with other proteins, with lipids, carbohydrates, water, minerals and flavours in a food system. Knowledge of these interactions could be of substantial benefit as a basis for the expansion of gluten and modified gluten applications.

For non-food applications, gluten is largely used in pet food, as it is a relative low cost ingredient and a good protein source. It is also used in binding heavy metals in industrial processes, removing ink from waste paper and in solidifying waste oil. Gluten and modified gluten have been used as aquaculture feed and calf-milk replacements (Day et al., 2006). Due to its thermoplasticity and good film-forming properties, gluten has been used in adhesives, paper coatings, medical bandages (Batey, 2004), slow-release encapsulation of pest- and weed-control agents (Quimby et al., 1994) and biodegradable films (Guilbert et al., 2002). Peptides from gluten are useful in cosmetics, lotions, and hair preparations, including products for skin firming and moisturising creams, as well as in biodegradable resins and in hair and facial cleansers (Day et al., 2006).

Recently, in a Europe-based project, wheat gluten was identified as a potential biopolymer for the production of renewable and biodegradable materials (FAIR-CT96-1979, 2000). Biomaterials having a variety of mechanical properties can be prepared from native or deamidated gluten and also from gliadin- and glutenin-enriched fractions, using either an aqueous casting procedure or thermomoulding. Generally, the differences in mechanical properties induced by the film-preparation process are greater than those arising from variations of protein composition. The gluten biomaterials are characterised as demonstrating a diverse range of elongation characteristics but with limited tensile strength. The European report on potential biomaterials concluded that diverse properties of gluten render it suitable to a range of specific novel industrial applications.

2.6 Summary of literature review on the production and applications of wheat gluten

Commercially, wheat gluten is current produced from flour using either the Martin or Batter process. Gluten initially was a by-product from the starch-separation process. Later on, it became a food ingredient due to its unique rheological properties. Gluten is primarily used for flour fortification in milling process and as a supplement to enhance the quality of bakery products. Gluten also has many applications in other food products including processed meat, breakfast cereals, extruded snacks and fruit bars. For non-

food usage, in addition to pet food, gluten is involved in processing of many other products including adhesives, paper coating, medical bandages and biodegradable packaging. As gluten has more applications in food and non-food industries and the demand for gluten has increased, the production of gluten has grown during the past ten years. One of the strengths of gluten as an ingredient is its relatively low price. The low water-solubility of gluten has limited its applications, but this could be overcome by chemical modification to render it more water soluble. Modified glutens have been produced as possible replacements for other protein ingredients. The functional properties of modified glutens are related to their interactions with other components in food systems; therefore, elucidating these interactions is likely to facilitate the usage of gluten in both food and non-food industries.

Chapter 3

Background and literature review: Wheat gluten protein - classification and analysis

The purpose of this chapter is to provide background and review the relevant scientific literature on wheat gluten protein. The areas covered are general information of amino acids, peptides and proteins; classification and structure of wheat proteins; bonding in dough formation and gluten structure; and some analytical methods for wheat protein fractionation and characterisation.

3.1 General information of amino acids, peptides and proteins

3.1.1 Amino acids: structure and classification

Proteins are natural polymers found in living organisms and composed of amino acids linked together via amide bonds, also known as peptide bonds (Figure 3.1). There are over 20 amino acids present in proteins and the different side chains result in varied molecular masses, solubilities, hydrophobicities and isoelectric points (pI) (Table 3.1).

Amino acids consist of an α-carbon atom covalently attached to a hydrogen atom, an amino group ($–NH_2$), a carboxyl group (–COOH) and a side chain R group (Figure 3.1). The physicochemical properties of amino acids are dependent on the chemical nature of the R groups. Amino acids with aliphatic (Ala, Ile, Leu, Met, Pro, and Val) and aromatic side chains (Phe, Trp, and Tyr) are hydrophobic as their solubilities in water are low (Table 3.1). Hydrophilic amino acids containing a polar side chain R group are soluble in water. They are either charged (Arg, Asp, Glu, His, and Lys) or uncharged (Ser, Thr, Asn, Gln, and Cys). The side chains of Arg and Lys contain guanidine and amino groups, respectively, and thereby are positively charged at neutral pH. The imidazole group of His is basic in nature. However, at neutral pH, its net charge is only slightly positive. The side chains of Asp and Glu acids contain a carboxyl group and thus they carry a negative charge at neutral pH. Both the basic and acidic amino acids

are strongly hydrophilic. The net charge of a protein is dependent on the relative numbers of basic and acidic amino acid residues in the protein (Damodaran, 1996).

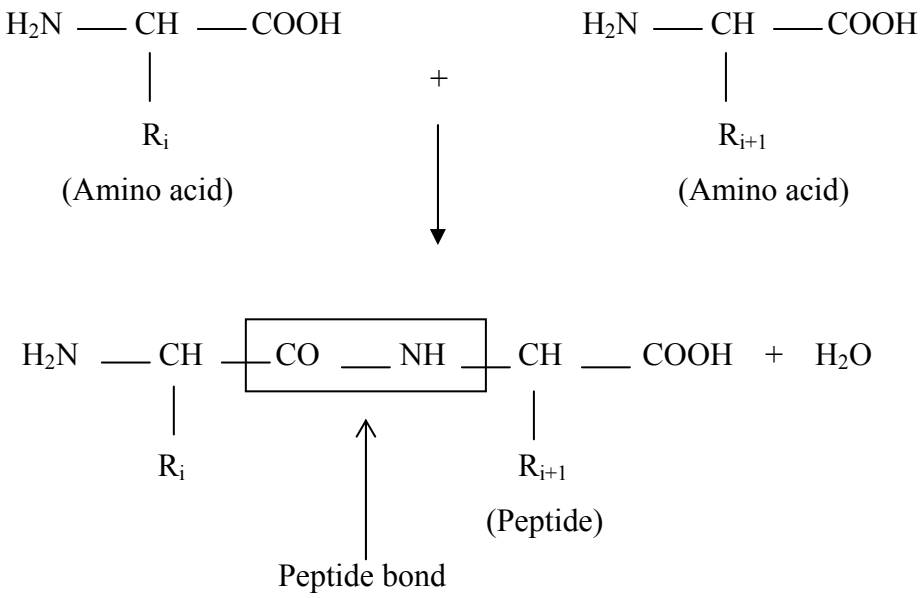


Figure 3.1 Amide (peptide) bond formation between amino acids

3.1.2 Bonding and structure of proteins

Amino acids are covalently linked via the peptide bonds in a linear sequence to form the backbone of the polypeptide as the primary structure of the protein chain. The peptide bonds result from the condensation of a carboxyl group (–COOH) and an amine group ($–NH_2$) with the removal of a water molecule (Figure 3.1). The terminus with the free α-amino group is known as the N-terminal, and that with the free α-COOH group is known as the C-terminal. In addition to the peptide bonds and primary amino-acid sequences, there are many interactions occurring at a molecular level and these determine the various structural attributes of proteins at the secondary, tertiary and quaternary levels.

The forces contributing to secondary, tertiary and quaternary structures are primarily intramolecular interactions. They include van der Waals and steric interactions formed between protein molecules as well as hydrogen bonding and both electrostatic and hydrophobic interactions that can be affected by the surrounding solvent (Damodaran,

1996). Moreover, the sulfhydryl groups (−SH) on cysteine residues can react with other cysteines to form disulfide bonds. This type of bond may make crosslinks between cysteines of the same (intramolecular bonding) or different (intermolecular bonding) protein chains (Cornell and Hoveling, 1998d).

Table 3.1 Primary amino acids occurring in proteins

Amino acids	Abbreviations	Molecular weight (MW)	pI	Solubility (g/L)*
Alanine	Ala (A)	89.1	6.00	167.2
Arginine	Arg (R)	174.2	10.76	855.6
Asparagine	Asn (N)	132.1	5.41	28.5
Cysteine	Cys (C)	121.1	5.07	-
Glutamine	Gln (Q)	146.1	5.65	7.2 (37°C)
Glycine	Gly (G)	75.1	5.98	249.9
Isoleucine	Ile (I)	131.2	6.02	34.5
Leucine	Leu (L)	131.2	5.98	21.7
Methionine	Met (M)	149.2	5.74	56.2
Phenylalanine	Phe (F)	165.2	5.48	27.6
Proline	Pro (P)	115.1	6.30	1,620
Serine	Ser (S)	105.1	5.68	422.0
Threonine	Thr (T)	119.1	5.68	13.2
Tryptophan	Trp (W)	204.2	5.89	13.6
Tyrosine	Tyr (Y)	181.2	5.66	0.4
Valine	Val (V)	117.1	5.96	58.1
Aspartic acid	Asp (D)	133.1	2.77	5.0
Glutamic acid	Glu (E)	147.1	3.22	8.5
Histidine	His (H)	155.2	7.59	-
Lysine	Lys (K)	146.2	9.74	739.0

Notes: *Solubility of amino acids in water, determined at 25°C, unless otherwise stated
Source: (Damodaran, 1996)

Two types of secondary structure show regular patterns, namely, helical and extended sheet-like structures. Helixes form when the angles of consecutive amino-acid residues

are twisted at the same set of dihedral angles on N–C_α and C_α–C bonds. These structures are stabilised by hydrogen bonds formed between the N−H on the backbone with the C=O group of the fourth preceding residue. In the sheet-like structure, the side chains are oriented perpendicularly (above and below) to the plane of the sheet; therefore, hydrogen bonding is possible only between segments and not within a segment (Damodaran, 1996).

The tertiary structure is the spatial arrangement obtained when a linear protein chain with secondary-structure segments folds further into a compact three-dimensional form. The formation of tertiary structure involves optimisation of various interactions including hydrophobic, electrostatic, van der Waals and hydrogen bonding to reduce the free energy of the molecule to a minimal value. In most cases, the hydrophobic residues are relocated to the interior of the protein structure while the hydrophilic residues, and especially charged residues, appear at the protein-water interface (Damodaran, 1996).

The quaternary level of structure refers to the spatial arrangement of a protein when it contains more than one polypeptide chain. Proteins can exist as dimers, trimers, tetramers, etc, termed oligomers, which may be made up of protein subunits (monomers) that are the same (homogeneous) or different (heterogeneous). The formation of oligomeric structures is the result of specific protein-protein interactions involving hydrogen bonds as well as both hydrophobic and electrostatic interactions. This structure formation is primarily driven by the thermodynamic requirement to bury exposed hydrophobic surfaces of subunits. However, it is physically impossible to bury all of the non-polar residues in the protein structure when the hydrophobic amino acid content of a protein is greater than 30%. Consequently, there is a greater probability for the interaction of hydrophobic patches between adjacent monomers to form quaternary structures (Damodaran, 1996).

3.2 Wheat gluten proteins: Classification and structure

Gluten protein is believed to be primarily responsible for the formation of strong cohesive dough from wheat flour. Gluten proteins are the predominant proteins in flour and they are concentrated in the endosperm of the wheat grain, comprising approximately 85% of the total endosperm protein (Cornell and Hoveling, 1998c). The

amino acid composition of these proteins is different to that of total wheat protein and flour (Table 3.2). Generally, they are low in the basic amino acids, such as lysine, and rich in proline and amide nitrogen (particularly glutamine). Hence, the gluten proteins have essentially no potential negative charges and only low levels of potential positive charge. As a result, they carry a low charge density on their molecules (Hoseney, 1998b).

Table 3.2 Amino acid composition of wheat, flour and gluten

Amino acid or ammonia	Wheat[a]	Flour[a]	Gluten[b]
Lysine	2.8	2.0	1.1
Histidine	2.4	2.1	2.0
Arginine	4.4	3.2	3.1
Aspartic acid	4.9	3.8	2.5
Threonine	2.8	2.6	2.1
Serine	4.5	4.5	3.4
Glutamic acid	32.3	35.4	36.7
Proline	10.6	11.7	13.0
Glycine	4.0	3.4	2.6
Alanine	3.5	2.9	2.1
Cysteine	2.4	2.5	1.4
Valine	4.2	4.1	4.4
Methionine	1.2	1.2	1.5
Isoleucine	3.4	3.6	3.7
Leucine	6.7	6.7	6.5
Tyrosine	1.7	1.4	3.2
Phenylalanine	4.6	4.8	4.6
Ammonia	3.6	4.0	5.0

Notes: (a) Amino acid composition expressed as g/100 g protein (Hoseney, 1998b)
(b) Amino acid composition expressed as % of total amino acids (Lookhart and Bean, 2000)

Proteins in wheat have been characterised in three ways: by their solubilities (Osborne, 1924), their electrophoretic mobilities (Shewry, 2003) and by their amino-acid

sequences (Tatham, 1995). These classification approaches are summarised in Figure 3.2.

3.2.1 Classification based on the solubility

Based on solubility, wheat proteins are classified into four groups: water-soluble albumins, salt-soluble globulins, alcohol-soluble gliadins, and acid- and alkali-soluble glutenins (Osborne, 1924). The first two groups comprise mainly metabolic proteins while the third and fourth groups consist largely of the gluten proteins. The alcohol-soluble gliadin group is characterised by a high content of proline and glutamine. This type of classification is useful for experimental work, although it is difficult to obtain reproducible results with fractional extraction, because the fractions obtained are not clear-cut, containing a mixture of subgroups and none of the solubility fractions contains a single pure protein.

3.2.2 Classification based on molecular weight and electrophoretic mobility

In general, gluten proteins can be classified as prolamin, mainly consisting of glutenins and gliadins. The distinction between gliadins and glutenins is based on their different functional properties, the glutenins being polymeric and the gliadins being monomeric (Batey, 2004). Glutenins are responsible for the elasticity of the gluten complex, which is due to the extensive network of intermolecular disulfide bonds arising from cysteine residues. Gliadins have a higher content of proline residues than glutenins and being much smaller monomeric proteins, they do not display such strong viscoelastic behaviour as the glutenin polymers.

The individual subunits of glutenins were originally classified based on their apparent sizes and mobilities on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) following reduction of disulfide bonds (Figure 3.2). They can be further described as belonging to one of four groups based upon their subunit structure, these groups being designated A, B, C and D (Shewry, 2003). The A subunits have apparent masses above 100 kilo Daltons (kDa) termed as high-molecular-weight (HMW) subunits of glutenin. The B and C groups of subunits have similar masses to α-, β-, γ-gliadins referred to as low-molecular-weight (LMW) subunits. The B group of subunits

is present in large quantities and migrates on SDS-PAGE with apparent MW ranging from 40 to 50 kDa, while the C group has the range of apparent MW of approximately 30 to 40 kDa (Lew et al., 1992). The minor group of LMW subunits (D) were identified with a slightly slower migration on SDS-PAGE than the B subunits (Jackson et al., 1983) and found to belong to the S-poor prolamins or ω-gliadins (Masci et al., 1991).

The standard classification used for gliadin subunits is based on their electrophoretic mobility at low pH (Figure 3.2). Low pH electrophoresis of gliadins resolves four groups of bands which are α-gliadins (fastest), β-gliadins, γ-gliadins and, finally, ω-gliadins (slowest) (Jones et al., 1959; Woychik et al., 1961). It has been found that the amino acid sequences of α- and β-gliadins are closely related and some gliadins with α-type sequences can appear in the γ-gliadin region of the electrophoresis gel (Kasarda et al., 1987).

3.2.3 Classification based on the amino acid sequence

On the basis of their amino acid sequences, the prolamins of wheat can be classified into three groups, referred to as HMW prolamins, S-rich prolamins and S-poor prolamins (Figure 3.2). These correspond to the HMW subunits of glutenin (HMW-GS), the α/β-,γ- and LMW subunits of glutenin (LMW-GS), and the ω-gliadins, respectively (Tatham, 1995; Shewry, 2003).

3.2.3.1 High molecular weight prolamins

The HMW-GS have actual MW ranging from 67 to 88 kDa (based on amino-acid sequences), but calculations based on their mobility on SDS-PAGE give apparent MW in the range of 82–115 kDa (Payne et al., 1981). All are rich in glutamine (~35 mol%), glycine (~20 mol%) and proline (~10 mol%) (Shewry et al., 1992). They consist of three domains; a central repetitive domain varying in length from 440 to 680 residues, flanked by non-repetitive N- and C-terminal domains of 81 to 104 and 42 residues, respectively (Tatham, 1995). The N- and C- terminal domains contain most or all of the cysteine residues. HMW-GS can be divided into low MW x-types and high MW y-types, based on the difference in their sequences and N-terminal domains. The x-type

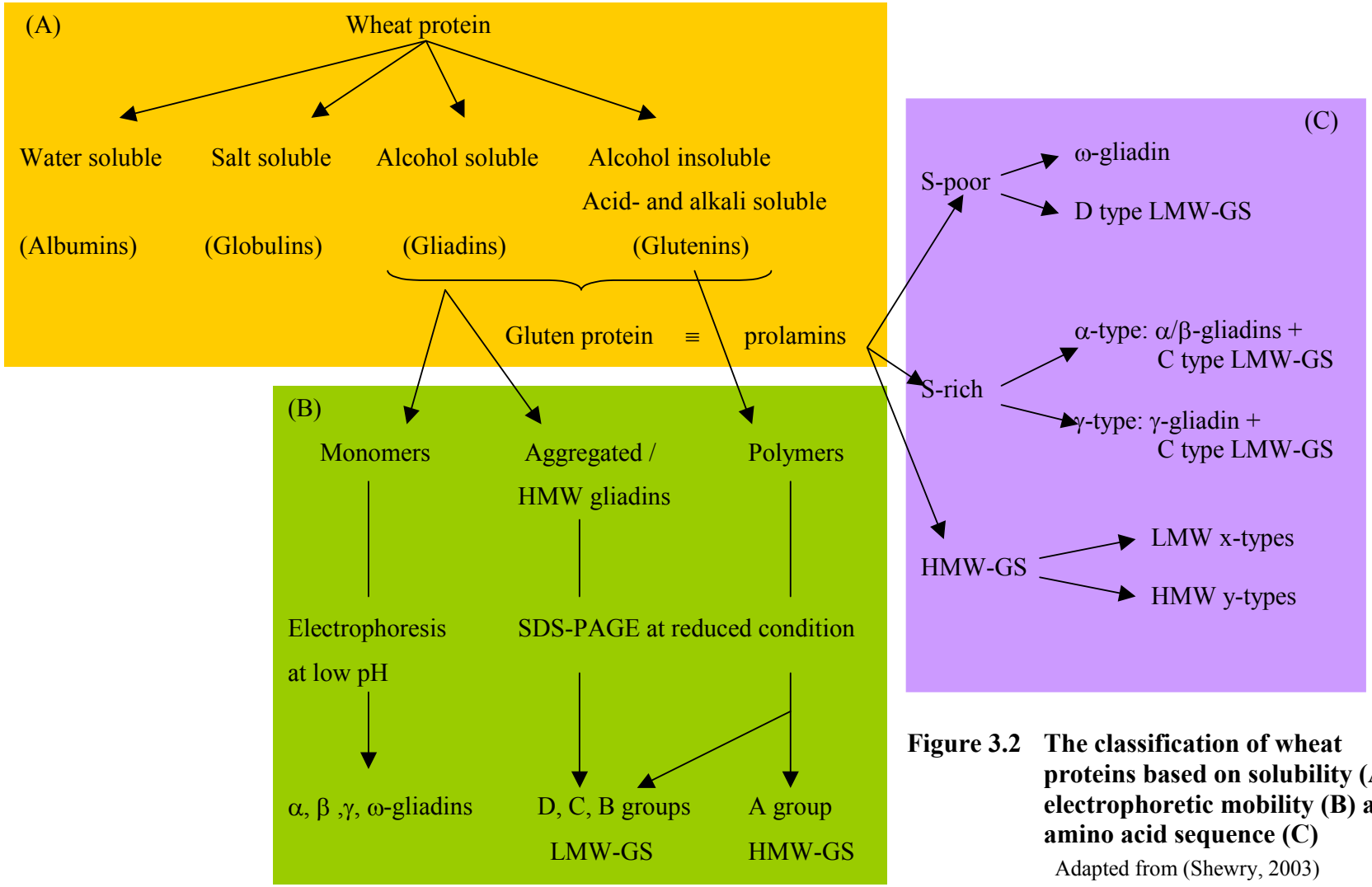


Figure 3.2 The classification of wheat proteins based on solubility (A), electrophoretic mobility (B) and amino acid sequence (C)

Adapted from (Shewry, 2003)

contains hexa-, nona- and tripeptide motifs while the y-type has only hexa- and nonapeptides. There are between four and seven cysteine units, with three (x-type) or five (y-type) occurring in the unique N-terminal domain and one in the C-terminal domain.

The secondary structures of the HMW-GS consist predominantly of overlapping β-turns, with an absence of α-helix or β-sheet structure. In the developing seed and in the dough systems, the proteins exist as hydrated solids, in contact with water and other components particularly starch and lipid. The hydrated functional gluten contains a considerable amount of intermolecular β-sheet in addition to α-helix or β-turn structures (Pezolet et al., 1992). The elasticity of gluten may result from intermolecular interactions involving these sheet structures. In the dry state of the HMW-GS, there is no evidence of β-sheet structure; increasing hydration leads to an increase in intermolecular β-sheet and extended chain structures. Upon hydration, there is formation of hydrogen bonds with other glutamine residues and the backbone of the protein. These structures may affect the elasticity of HMW-GS in gluten and dough systems. The glutenin polymers have MW extending up into the tens of millions; the HMW-GS are present in greater proportions in glutenin polymers having MW in excess of 1×10^6. These polymers are stabilised primarily by intermolecular and also by intramolecular disulfide bonds.

3.2.3.2 S-rich prolamins

The S-rich prolamins (Figure 3.2) have MW by SDS-PAGE of about 30−45 kDa and characterised by high levels of cysteine (2−3 mol %), proline (15−18 mol %) and glutamine (37−40 mol %). They are the major group of prolamins present in wheat and can be broadly classified into three groups α/β-gliadins, γ-gliadins and LMW-GS (Tatham, 1995). They all consist of two distinct structural domains including the N-terminus with repeating glutamine and proline and the C-terminus with non-repetitive residues and primarily containing cysteine residues. Molecules of α-/β- and γ-gliadins are globular and can be stabilised by hydrogen bonding with a contribution from ionic and hydrophobic interactions. They are the most diverse in structure and consist of monomeric proteins containing intra-chain disulfide bonds (gliadins) and polymeric

proteins (LMW-GS) with both intra-chain and inter-chain disulfide bonds (Shewry and Tatham, 1997).

The LMW-GS are divided into three groups B, C and D. The B-type of LMW-GS form a discrete group with two sub-classes called LMWs and LMWm on the basis of their terminal sequences (Lew et al., 1992). The C-type are related to the α- and γ-type gliadins, with additional cysteine residues which contribute to inter-chain disulfide bond formation with cysteines in HMW-GS and other LMW-GS (Lew et al., 1992; Shewry and Tatham, 1997; Anderson et al., 2001). The D-type appear to be similar to the ω-gliadins but have more cysteine residues (Masci et al., 1991).

All γ-gliadins contain eight conserved cysteine residues that are located in the C-terminal domain and form four intra-chain disulfide bonds (Shewry, 2003). Six of these cysteine residues that form three disulfide bonds are also present in the α-gliadins (cysteines 1,4,5,6,7,8) and the B-type of LMW-GS (cysteines 1,2,3,4,5,7). One or more additional cysteines present in the B-type of LMW-GS can participate in the formation of inter-chain disulfide bonds (Shewry and Tatham, 1997).

3.2.3.3 S-poor prolamins or ω-gliadins

The ω-gliadins make-up about 10% of the total prolamins of wheat, consisting almost solely of repetitive sequences, with only short N- and C-terminal regions (Shewry, 2003). They lack cysteine residues and thus are unable to form either intra- or inter-chain disulfide bonds. ω-Gliadins interact with other components of gluten and doughs particularly through hydrogen bonds. Comparisons of amino acid compositions and N-terminal amino acid sequences indicate that most ω-gliadins have similar structures (Tatham and Shewry, 1995), except those encoded by the B genome which have a different consensus repeat motif (DuPont et al., 2000). The ω-gliadins encoded by chromosomes 1A and 1D contain high contents of glutamine (40 mol%), proline (30 mol%) and phenylalanine (9 mol%), whereas levels of amino acids in the 1B encoded ω-gliadins are relatively high for glutamine (50 mol%), lower for proline (20 mol%) and similar in the case of phenylalanine (Tatham, 1995).

In the secondary structure, ω-gliadins are primarily comprised of β-turn and small proportions of α-helix or β-sheet structures. In the dry state, the backbone of ω-gliadins appears to be distorted by extensive hydrogen bonding involving the glutamine side-chains. With increasing water content, these hydrogen bonds may be broken by water molecules, leading to more intermolecular β-sheet structures. At high levels of hydration and in solution, β-turns and extended chain structures predominate (Tatham, 1995). In glutens and doughs the ω-gliadins can form hydrogen bonds with other prolamins, not being covalently bound into higher polymers, they are able contribute to the viscous flow of gluten and dough.

3.3 Lipid-binding proteins

Lipid-binding proteins are defined as all proteins that in their native folding structure are capable of spontaneously binding lipids. They are normally identified by the co-existence with lipid in their fractions. However, the presence of lipids in the protein fractions does not conclusively prove the formation of complexes of lipid and protein. It could be due to their solubility, extractability and the structural changes of proteins in the solvents. Therefore, other evidences are essential to confirm the lipid-binding properties of the proteins under study.

Many lipid-binding proteins, particularly purothionin, ligolin, S protein, chloroform/methanol soluble (CM) proteins, puroindolines (PIN) and lipid-transfer proteins (LTP), have been isolated from wheat. The characteristics of these proteins will be discussed in the following sections. The lipid-binding properties of these proteins are covered in Chapter 5.

3.3.1 Purothionin, ligolin, S protein and CM protein

Purothionins, isolated from a light petroleum extract of flour, have been found to be predominantly associated with polar lipids (Redman and Fisher, 1968; Hoseney et al., 1970b). They show two closely migrating bands on gel electrophoresis, designated α- and β-purothionins (Marchylo et al., 1976). The amino-acid composition of purothionins is markedly different from that of most flour proteins as they are rich in

cysteine (approximately 20%) as well as lysine and arginine (totalling approximately 20%), but low in glutamine and proline (Lasztity, 1996).

Another lipid-binding protein, named as ligolin, was isolated from the final supernatant in the selective precipitation of AUC (0.1 M acetic acid, 3 M urea, 0.01 M cetyltrimethylammonium bromide) soluble gluten protein with ammonium sulfate (Frazier et al., 1981). Ligolin is characterised by a 9 kDa MW peak during fractionation on a Sephadex G50SF gel-filtration column. The amino-acid composition of this protein is different from that of glutenin and purothionin (Frazier et al., 1981), while sharing some characteristics with wheat LTP (Marion et al., 1998). They are higher in aspartic acid and lower in glutamic acid than glutenin. The contents of cysteine, lysine and arginine are higher in ligolin than in glutenin but not as high as in purothionin (Frazier et al., 1981). Ligolin is similar to wheat LTP with respect to the absence of methionine and the presence of two tyrosines; however, in contrast to ligolin, LTP does not contain phenylalanine and is different to ligolin in the amounts of leucine, glutamic acid, threonine and histidine (Marion et al., 1998).

S proteins have been fractionated using a similar method to that reported by Frazier et al. (1981) and these have been found to associate with polar lipids (Zawistowska et al., 1985). The S protein fraction was characterised with three subfractions consisting of subfractions I, II and III on a Sephadex G-50 gel-filtration column. Subfraction III comprised proteins with MW of 14 and 16 kDa on SDS-PAGE while subfractions I and II contained many components with MW above 16 kDa. It was also found that the 25 kDa and lower MW proteins were the major components of the subfraction II. The most abundant amino acids in subfraction III were cysteine (12%), glutamic acid (11%), proline (10%), glycine (9%) and basic amino acids (9%). The amount of subfraction III was higher in wheat varieties having lower processing potential, with slightly higher lipid content (Zawistowska et al., 1986). Moreover, in characterising the S proteins using two-dimensional electrophoresis (2DE), these were found to be almost identical to CM proteins (Zawistowska and Bushuk, 1986).

Lipoproteins extracted by solutions of chloroform and methanol consist of the CM proteins, LMW gliadins and some other gliadins (Meredith et al., 1960; Lasztity, 1996). CM proteins have a MW of 12 to 13 kDa and they contain high proportions of

hydrophobic amino acids. They also comprise lower amounts of glutamine and higher amounts of lysine compared to typical gliadins (Lasztity, 1996). LMW gliadins have a MW of 16 to 19 kDa and are high in glutamine (23−27%) and proline (9.1−11.4%) and low in lysine (0.0−0.3%). Furthermore, the CM proteins have been shown to be components of the tetrameric α-amylase inhibitor in wheat (Sanchez-Monge et al., 1986). In comparisons based on the inhibitory activities on α-amylases and proteases, amino-acid composition, MW and isoelectrofocusing (IEF) patterns, the LMW proteins (extracted from wheat flour using either AUC-ammonium sulfate or 70% ethanol) are found to be similar to the S protein fraction, ligolin and the CM proteins, but different to purothionins (Prieto et al., 1993).

3.3.2 Puroindolines and lipid-transfer proteins

Two other types of lipid-binding protein found in wheat flour are the family of plant non-specific LTP and PIN. Non-ionic or zwitterionic detergents are frequently used to isolate these proteins. The detergents partition into membrane bilayers and lead to the formation of mixed micelles composed of lipids, proteins and detergent molecules (Helenius and Simons, 1975). Two strategies have been used to determine the lipid-binding properties, based on a lipid-binding assay (using either radio-labelled or fluorescent lipids) or on the structural features of protein and lipid aggregates (using far-UV circular dichroism (CD)) (Kooijman et al., 1997).

Triton X114 (TX114) has been used to extract the LMW proteins from flour and these proteins have been characterised as a mixture of purothionins and a protein similar to ligolin and CM proteins (Blochet et al., 1991). From the TX114 extract, a new group of closely related proteins was subsequently identified and named as PIN. Following purification, the sequences were determined (Blochet et al., 1993) and found to contain 10 cysteine residues forming five disulfide bridges. These proteins have a mean MW of 12.8 kDa and are basic proteins with a calculated pI of 11. Two different amino sequences were identified, demonstrating a unique trytophan-rich domain with Trp-Arg-Trp-Trp-Lys-Trp-Trp-Lys at position 38−45, named as puroindoline-a (PIN-a) and a truncated trytophan-rich domain with Trp-Pro-Thr-Lys-Trp-Trp-Lys at position 39−45 referred to as puroindoline-b (PIN-b) (Kooijman et al., 1997).

LPTs primarily occur as non-specific forms termed as non-specific lipid-transfer proteins (nsLTP). They can be extracted from wheat flour or bran using deionised water and then enriched using cation-exchange chromatography (Charvolin et al., 1999). Plant nsLTP are LMW proteins, containing eight cysteine residues forming four disulfide bridges. Two main families have been identified as LTP1 and LTP2 with MW of 9 and 7 kDa, respectively (Douliez et al., 2000a). Both LTP1 and LTP2 are found in cereal seeds and primarily concentrated in the aleurone layer. From the amino acid sequence, the cysteine pairing, Cys10-Cys24, Cys25-Cys60, Cys2-Cys34, Cys36-Cys67, revealed a mismatch at the Cys34-X-Cys36 motif of LTP2 compared with LTP1. LTP1 and LTP2 contain similar cysteine pairing except a mismatch at the Cys-X-Cys motif (Figure 3.3).

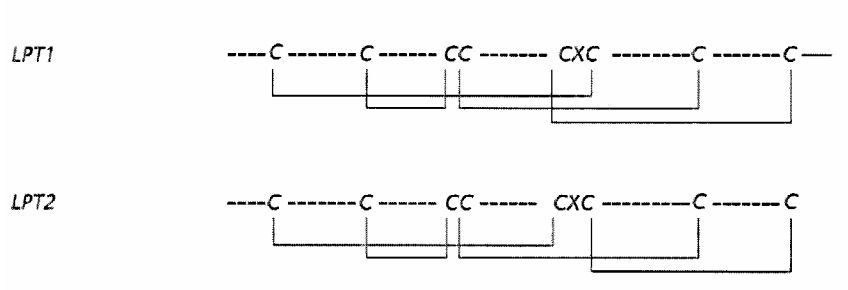
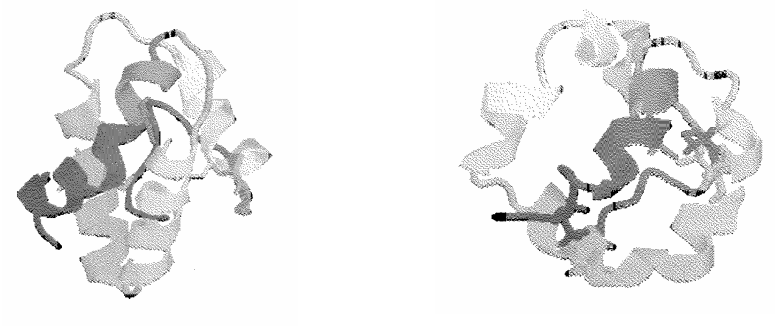


Figure 3.3 A difference on a motif of amino acid sequence of LTP1 and LPT2
Source: (Marion et al., 2003)

Both LTP1 and LTP2 are α-helical proteins, in which four helices of LTP1 form a bundle-like shape surrounded by a C-terminal polypeptide whilst LTP2 displays three main helices and two single-turn helices forming a rigid and unique structural motif (Figure 3.4) (Marion et al., 2003). Although the secondary structure of LTP2 is close to that of wheat LTP1, lipid-transfer activity of LTP2 is at least fivefold higher than that of LTP1 (Douliez et al., 2001c).

Two isoforms of LTP2 have been isolated and named as LTP2 (G) and LTP2 (P), which have a slight difference in MW, 6,971 and 7,038, respectively. The difference is due to the replacement of proline found as amino acid 33 in LTP2 (P) with glycine in LTP2 (G) (Douliez et al., 2001c).



LTP1 LTP2

Figure 3.4 Structural characteristics of LTP1 and LTP2
Source: (Marion et al., 2003)

Recently, a new isoform called LTP1b was isolated from wheat seeds (Douliez et al., 2001a) and its MW was reported to be 294 Da higher than the native LTP1. LTP1 and LTP1b cannot be differentiated by SDS-PAGE but can be separated by C-18 RP HPLC eluted with 33% acetonitrile (Douliez et al., 2001a). LTP1 and LTP1b were found to have similar N-terminal sequences. They were able to transfer lipids with the same efficiency and could not be distinguished on the basis of either absorption spectrum or far-UV CD spectra. In addition, they exhibited the same typical maximum fluorescence at 305 nm, however, tyrosine fluorescence indicated that the intensity for LTP1b was 2-fold lower than that of LTP1.

A fatty-acid-binding protein (W-FABP) of approximately 7 kDa has been isolated from mature wheat kernels by water extraction and gel filtration of the extract, followed by two steps of HPLC (Castagnaro and García-Olmedo, 1994). The N-terminal amino acid sequence of this protein was determined up to the 28th residue and could be classified as a nsLTP. As found for other LTP, W-FABP does bind fatty acids, although it has other characteristics which are quite different, as is not recognised by LTP antibodies and the pI value is more acidic (6.8) compared with that of LTP (9.6).

3.4 Bonding involved in dough formation and gluten matrix integration

Dough formation begins when flour comes into contact with water. During mixing, glutenin interacts with gliadin to form gluten, which is the viscoelastic matrix of the dough. This is a highly complex chemical system as it is produced from wheat flour that

contains many different components including starch, proteins, lipids, fibre and minerals. Water addition and mechanical mixing facilitate the interactions of the various components, with those occurring between protein components being the focus in this Section whilst protein-lipid interactions are reviewed in Chapter 5.

Many chemical bonds are involved in dough development and these have been thoroughly reviewed by Pomeranz (1987); Bushuk (1998); Wrigley et al. (2006). Two types of covalent bonds contribute to the backbone of proteins and they are peptide linkages between amino acids and disulfide links within and between peptide chains. These bonds are strong and require high energy to be broken, whereas non-covalent bonds, consisting of ionic, hydrogen and hydrophobic bonds, are much weaker providing flexibility to protein structures (Table 3.3).

Table 3.3 Chemical bonds in dough

Bond	Energy (kcal.mol^{-1})	Mobility
Covalent	30−100	Nil
Ionic	10−100	Medium
Hydrogen	2−5	High
Hydrophobic	1−4	High
Van der Waals	0.5	High

Source: (Bushuk, 1998)

In dough formation, the most important bonds are the hydrogen and hydrophobic interactions, disulfide bonds and possibly dityrosine crosslinks (Tilley et al., 2001). Disulfide bonds play a key role in the formation and development of dough. They form strong links within and between polypeptide chains, therefore stabilising hydrogen bonds and hydrophobic interactions. Disulfide bonds can be mobilised through disulfide-interchange reactions, which involve sulfhydryl-containing soluble or LMW proteins (Wrigley et al., 2006).

The ability to form disulfide bonds varies between the various types of proteins present in the flour (Shewry and Tatham, 1997; Wrigley et al., 1998). For example, ω-gliadins

lack sulfur-containing amino acids and cannot form disulfide bonds. Although the α-, β- and γ-gliadins have sulfhydryl groups, they do not contain free cysteine residues in their structure to form the link between these gliadins. The disulfide cross-links of these proteins are intra-chain and their role is in stabilisation of the folded conformation (Shewry and Tatham, 1997). Therefore, all gliadins are present in the monomeric form. LMW-GS and HMW-GS contain many sulfhydryl groups that contribute to intra-chain linkages as well as to those involving other glutenin subunits. Thus, extensive disulfide cross-links occur in glutenin, resulting in the formation of polymeric proteins having large MW (10^5–10^7 Da) and these cross-links in the coil form of glutenin polypeptides contribute to the resistance to extension of dough (Wrigley et al., 1998).

Hydrogen bonds are formed between hydrogens within hydroxyl, amide, or carboxyl groups and oxygen units present in carboxyl groups. As a significant number of hydrogen bonds are formed between small molecules, the viscosity of a liquid will tend to increase and in a dough system they will affect mobility and plasticity. Although hydrogen bonds are much weaker than covalent bonds, they still contribute significantly to the structure of dough, due to the large number of bonds. Moreover, hydrogen bonds are able to interchange under stress and thereby facilitate the re-orientation of protein chains, and allow for stress relaxation of structural-activated doughs (Bushuk, 1998).

Hydrophobic bonds result from the interactions of non-polar groups in the presence of water. The involvement of hydrophobic interactions in dough is related to the prevalence in the proteins of amino acids with non-polar side chains (Bushuk, 1998). Approximately 35% of the amino acid residues of gluten are hydrophobic. Therefore, the hydrophobic interactions between gluten proteins can play an important role in stabilising gluten structure and in the lipid-binding properties of gluten, as polar residues cannot exist in the hydrophobic core of the protein (Hoseney, 1998b). The hydrophobic property of gluten protein can sustain the intra- and intermolecular hydrophobic bonds. The nature of their contribution in dough development is similar to that of hydrogen bonds but the extent is less significant (Wrigley et al., 2006). The bond energy of hydrophobic bonds is sufficiently low (Table 3.3) to facilitate rapid interchange at room temperature, thereby contributing to dough plasticity (Pomeranz, 1987).

3.5 Wheat protein fractionation

Wheat proteins are a complex mixture of related polypeptides. The first attempted fractionation of wheat proteins applied a sequential extraction using water, salt solution and 70% ethanol to separate albumin, globulin, gliadin and glutenin from wheat flour, based on their respective solubilities (Osborne, 1924). However, the problem of this fractionation was the impurity of protein in each fraction. Many methods have been developed to enhance the fractionation of gluten proteins using different solvents or mixtures of solvents; these are summarised in Table 3.4.

In a modification of the Osborne procedure, glutenins have been separated into soluble and insoluble glutenins using acetic acid (Chen and Bushuk, 1970; Bietz and Wall, 1975). Dimethyl sulfoxide and 70% ethanol was used to extract a glutenin fraction enriched in HMW-GS (Burnouf and Bietz, 1989). The combination of aqueous ethanol solution with various concentrations of reducing agent has been applied for the separation of HMW-GS and LMW-GS at a preparative level (Larré et al., 1997). The extraction of gliadins has been optimised by varying the concentration of ethanol aqueous solutions (Wieser et al., 1994). The use of propan-1-ol in conjunction with other chemicals including reducing agents (dithiothreitol, 2-mercaptoethanol, NaI), ethanol or salt has enhanced the separation of glutenins and gliadin (Byers et al., 1983; Marchylo et al., 1989; Fu and Sapirstein, 1996; Sapirstein and Fu, 1998; Fu and Kovacs, 1999; Suchy et al., 2003). These modifications have facilitated the separation of gluten proteins, although they involve many non-food grade chemicals, produce small quantities of the fractions and, therefore, are only suitable for analytical purposes.

At various dilute concentrations of hydrochloric acid or acetic acid, gluten proteins have been sequentially fractionated into many fractions containing different proportions of gliadin and glutenin (Cornec et al., 1994; MacRitchie, 1985; Berot et al., 1994). These fractionation procedures have the advantage that protein fractions are produced in large quantities suitable for reconstitution studies (MacRitchie, 1987).

Table 3.4 Approaches to the fractionation of gluten proteins

Protein separation	Sample	Solvent system	References
Water soluble protein / storage protein	Wheat grain	Water; 0.01−2.00 M NaCl solution; 70% ethanol; 50% propan-1-ol with and without 2-mercaptoethanol	(Byers et al., 1983)
Monomeric-rich protein / Soluble glutenin-rich protein / Insoluble gluten-rich protein	Flour	1) 7.5% propan-1-ol and 0.3 M NaI 2) 50% propan-1-ol 3) 40% propan-1-ol and 0.2% dithiothreitol	(Marchylo et al., 1989; Fu and Sapirstein, 1996; Sapirstein and Fu, 1998; Suchy et al., 2003)
Monomeric / polymeric protein	Flour	NaI (0.25−2.0 M, 0.25 M increments); propan-1-ol (5–50%, 5% increments); or combination of NaI and propan-1-ol	(Fu and Kovacs, 1999)
Glutenin enriched in HMW-GS	Flour	Dimethyl sulfoxide / 70% ethanol	(Burnouf and Bietz, 1989)
Albumin and globulin / gliadin / soluble glutenin / insoluble glutenin	Flour	0.5 M NaCl; 70% ethanol; 0.05 M acetic acid	(Chen and Bushuk, 1970)
Albumins/globulin and gliadins	Flour	0.4 M NaCl / 0.067 M NaK phosphate, pH 7.6 and 60% aqueous ethanol	(Wieser et al., 1994)
HMW-GS / LWM-GS	Gluten	50% ethanol with various ratios of dithiothreitol and protein	(Larré et al., 1997)
Gluten sub-fractions: monomeric / polymeric protein	Flour / Gluten	Dilute hydrochloric at various concentrations	(MacRitchie, 1985; Cornec et al., 1994)
Gliadin-rich fraction / glutenin-rich fraction	Gluten (dried)	Acetic acid at various concentrations	(Berot et al., 1994)
HMW-GS / LMW-GS	Flour	0.05 M borate buffer, pH 8.5, 2% 2-mercaptoethanol, 1 g/L glycine with various urea concentrations	(Nicolas et al., 1997)
Size of wheat protein	Flour	Flow Field-Flow Fractionation combined with sonication, sodium dodecylsulfate (SDS) and hydrochloric acid or acetic acid	(Stevenson and Preston, 1996; Wahlund et al., 1996)

Recently, flow field-flow (FFF) fractionation has been used to detect the presence in native polymeric glutenin of components with molecular masses as large as millions of Da (Stevenson and Preston, 1996). The combination of sonication and the use SDS during the preparation of protein samples and the FFF fractionation performed in the carrier buffer containing SDS have provided a rapid procedure for separation of unreduced glutenin (Wahlund et al., 1996).

The solubility of gluten proteins is an issue in the selection of an extraction or fractionation method. In the aqueous condition, gluten proteins tend to aggregate through non-covalent bonds, and many reagents including SDS, urea, dithiothreitol or 2-mercaptoethanol (Nicolas et al., 1997) have been used to increase the solubility. In addition, sonication is often used to enhance the extractability of very large glutenin polymers (Singh et al., 1990).

3.6 Wheat gluten protein analysis

Electrophoresis, chromatography, spectroscopy, and amino-acid analysis are common analytical techniques applied in studies of wheat gluten proteins. These techniques are primarily used to determine the variety of wheat and the quality of flour, to identify protein types in flour, gluten and protein fractions, or to verify the efficiency of fractionation methods. These approaches have been thoroughly reviewed and summarised by Bean and Lookhart (2003); Khan et al. (2003); Koehler (2003); Lookhart et al. (2003); Preston and Stevenson (2003).

3.6.1 Electrophoretic methods

Different electrophoretic methods including aluminium lactate/lactic acid-polyacrylamide gel electrophoresis (A-PAGE), SDS-PAGE, 2DE and capillary electrophoresis (CE) have been used to separate and characterise storage proteins of wheat (Bietz and Simpson, 1992; Bean and Lookhart, 2000; Bean and Lookhart, 2003; Khan et al., 2003).

3.6.1.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) has been widely used for separation of the proteins from all cereals. In principle, polyacrylamide gels provide a versatile, porous matrix that can be used to separate proteins on the basis of charge and/or size (Khan et al., 2003). The sizes of pores in acrylamide gels are inversely related to the concentration of acrylamide and dependent on the ratio of acrylamide to bis-acrylamide and the conditions of polymerisation. Variation of buffer systems and other additives, including detergent or reducing agent, contribute to the options available for the separation of complex molecules.

As gluten proteins are characterised by a low charge density, a high level of hydrophobicity and a large number of hydrogen bonds (Pomeranz, 1987; Bushuk, 1998; Wrigley et al., 2006), SDS, an anionic detergent, is used to disrupt hydrogen bonds, to block hydrophobic interactions, to unfold the protein molecules and to form a complex with proteins having a negative net charge. Under electrophoretic conditions, ionic species travel through the porous gel matrix that acts like a sieve to separate proteins based on molecular size. Apparent MW can be estimated from electrophoretic mobility by comparison with protein standards of known MW.

For wheat, SDS-PAGE has been used for the separation of HMW-GS (Payne et al., 1979). Due to a large range of MW of wheat proteins, multistacking gels at pH 6.9 of 4, 6, 8, 10 and 12 % (w/v) acrylamide concentration were developed to analyse a protein extract from dough under non-reduced and reduced conditions (Khan and Huckle, 1992). Specific methods have been developed to improve the separation of HMW-GS (Graybosch and Morris, 1990; Huang and Khan, 1998). Subsequently, precast gradient gels with NuPAGE system provided better resolution of HMW-GS (Kasarda et al., 1998). LMW-GS have been difficult to separate from gliadin due to the similarity in MW. Some methods have been developed to solve the problem in separating the HMW-GS and LMW-GS of wheat (Khelifi and Branlard, 1991; Zhen and Mares, 1992).

A-PAGE, based on the methods of Jones et al. (1959); Woychik et al. (1961); Bushuk and Zillman (1978); Lookhart et al. (1982); Khan et al. (1983), is used to separate gliadin proteins for the identification of wheat varieties. It is also used for the separation

of other monomeric proteins typically albumins and globulins. Most of PAGE separations of gliadins use pH 3.1 aluminium lactate acid buffer and a uniform gel. Variations in gel thickness, buffer type, pH, ionic strength, temperature, catalyst and apparatus design can be adjusted to optimise the gliadin separations (Khan et al., 1985; Lookhart et al., 1985). Gradient polyacrylamide concentration in A-PAGE can also reduce mobility of proteins and sharpen bands (Du Cros and Wrigley, 1979). A rapid A-PAGE performed with a precast gradient gel at pH 3.1 is useful in determining wheat varieties for commercial requirements (Wrigley et al., 1991). Glutenin subunits have also been successfully separated on acid-PAGE when adding urea in the acetic acid buffer solution, providing good resolution of glutenins (Morel, 1994).

3.6.1.2 Two-dimensional electrophoresis and mass spectrometry

2DE has provided extremely high resolution in the separation of proteins. In this technique, proteins are fractionated in the first dimension on the basis of charge and in the second dimension by the size (Khan et al., 2003). Therefore, IEF or nonequilibrium pH gradient electrophoresis (NEPHGE) to resolve proteins with pI greater than 7.5 is normally used for the first dimension and SDS-PAGE for the second dimension. In the field of cereal chemistry, 2DE was first applied to gliadin proteins (Wrigley, 1970). Since then, many different methods have been developed for enhanced resolution in the separation of wheat proteins (Payne et al., 1985; Dougherty et al., 1989).

With recent improvements in technology approximately 1,300 polypeptides were resolved on 2DE gel performed at two pH ranges: 4.0–7.0 and 6.0–11.0 and characterised on the basis of the N-terminal sequences (Skylas et al., 2000). Recently, 2DE (immobilised pH gradient (pH 6–11) and SDS-PAGE) was used to characterise amphiphilic proteins that are soluble in the non-ionic detergent TX114 (Amiour et al., 2002). A total of 446 spots were identified, and the results were discussed in relation to grain softness and dough properties.

Matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS) is a relatively newly developed technique, which can be used for analysing both gliadins and glutenin subunits (Dworschak et al., 1998). The combination of 2DE and MALDI-TOF allows characterisation of wheat proteins in relation to their genome

(Islam et al., 2003) and has demonstrated the diversity of puroindolines in wheat protein (Branlard et al., 2003).

3.6.1.3 Free-zone capillary electrophoresis

Free-zone capillary electrophoresis (FZCE) is the simplest and most often used mode of high performance capillary electrophoresis (HPCE). In this mode, capillaries are simply filled with separation buffer. Protein sample is introduced into the capillary using a pressure injection. When the voltage is applied, an electrical field is established inside the capillary, proteins migrate to the appropriate electrode and are detected by a UV detector. The separation of proteins in this method is based on differences in their charge density and is normally performed in acidic conditions, which is similar to the separation condition used for wheat proteins on A-PAGE gel.

The many buffer systems used to separate wheat protein on FZCE have been reviewed by Bean et al. (1998); Bean and Lookhart (2000). At various concentrations, a borate buffer system containing acetonitrile and the detergent SDS can be used to separate wheat gliadins. Better resolution has been achieved with the combination of small inner diameter capillaries and sodium phosphate based buffer (Lookhart and Bean, 1995). Optimal resolution and baseline were obtained with gliadins extracted with 30% ethanol. Reproducibility has been a problem for HPCE with the borate buffer system because the elution time of peaks was inconsistent upon repeated analyses. This could be due to low solubility of an aqueous buffer and the interaction or aggregation of gluten proteins in the capillary. A suitable procedure for conditioning and washing the capillary has been found to improve the repeatability of results.

A capillary coated to provide a positive charge, used in conjunction with an aluminium lactate buffer has been used to separate gliadin into 15 to 20 major peaks in less than 20 min (Werner et al., 1994). Phosphate buffer (pH 2.5) containing a polymeric additive (hydroxypropylmethylcellulose, HPMC) produces higher resolution and reproducibility for protein separation (Bean et al., 1998). The phosphate buffer system was then used to separate cereal proteins with various capillaries having inner diameters from 50 to 20μm (Lookhart and Bean, 1995). Different organic solvents and detergents in combination with a low pH phosphate buffer have been investigated to improve the

resolution of wheat protein on HPCE (Lookhart and Bean, 1996). This buffer system was also used to characterise the TX114 soluble protein (Blochet et al., 1993; Day and Schofield, 2001).

3.6.2 Chromatographic methods for wheat proteins

3.6.2.1 Reversed-phase high-performance liquid chromatography

RP-HPLC is the most common HPLC method used for gluten protein analysis. It is fast, reproducible, sensitive, quantifiable, and gives good recovery. It is suitable for preparative as well as analytical separations.

In RP-HPLC, the stationary phase is a silica based support modified with alkyl chains that provide a hydrophobic surface and the mobile phase is a solvent, commonly a mixture of acetonitrile with water. The principle of fractionation in RP-HPLC is based on the different surface hydrophobicities of proteins. After a solute is adsorbed to the stationary phase, the mobile phase is pumped through a column leading to the partitioning of the solute between the mobile and stationary phases (Bradshaw, 1998). This partitioning could be due to the competition of the solute and mobile phase for binding sites at an interfacial surface on the stationary phase. Therefore, the retention time is a function of the strength of the interaction between proteins and the hydrophobic groups on the stationary phase.

RP-HPLC method can be applied to analyse all types of wheat proteins by varying extraction conditions and gradients. A good separation of gliadins was achieved with a C-18 column, using a gradient of acetonitrile from 20 to 60% and 0.05−0.1% trifluoroacetic acid (Bietz, 1983) and an elevated temperature (50−70°C) improved the resolution (Bietz and Cobb, 1985).

Many applications of RP-HPLC methods in analysing gliadins and glutenin subunits have been reviewed by Lookhart et al. (2003). RP-HPLC was used to isolate and characterise sequentially extracted wheat protein in relation to dough strength (Kruger et al., 1988); to compare gliadins from flour mill streams and fractions (Lookhart et al., 1989); to study the relationship of protein composition and technological and rheological qualities (Wieser et al., 1994; Sadouki et al., 2005); to study the effect of

flour chlorination on wheat gliadins (Sinha et al., 1997); to quantify and determine prolamin groups (Nicolas et al., 1998); and to evaluate the extraction and fractionation methods by quantifying gliadins, glutenins and albumins/globulins in a single flour sample (DuPont et al., 2005).

3.6.2.2 Size-exclusion chromatography

Size-exclusion chromatography, also known as gel-filtration chromatography, has been the most widely technique used for the size characterisation of wheat proteins. Packing materials of a size-exclusion column for HPLC are commonly silica-based and consist of particles containing pores of well-defined size. As the mobile phase flows through the column, the solute migrates with the solvents. The separation is based on the interaction of proteins with the pores within the stationary phase. Small particles, particularly salts and small peptides which interact with the pores, migrate slowly through the column whilst the large peptides or proteins are excluded from the pores and therefore migrate through the column more quickly.

Most size-exclusion high performance liquid chromatography (SE-HPLC) studies on wheat protein have focused on the overall profiles of monomeric and polymeric proteins. Early studies (Bietz, 1985; Huebner and Bietz, 1985; Dachkevitch and Autran, 1989) showed that 0.1 M sodium phosphate buffer pH 6.9 containing 2% SDS could extract 55−90% of total flour protein for SE-HPLC analysis. Sonication improved the extraction of total protein in the absence of reducing agent (Singh et al., 1990). Heat treatment of protein extracts at about 60°C was essential to obtain consistent SE-HPLC results, which could be related to a denaturation of proteases (Larroque et al., 2000). SDS in the buffer gradually caused the deterioration of the column, thus a replacement of normal running buffer with 50% acetonitrile containing 0.1% trifluoroacteic acid resulted in an enhancement of peak resolution (Batey et al., 1991).

3.6.3 Amino-acid analysis

Amino-acid analysis provides invaluable information about protein composition, which can be used to identify specific proteins in conjunction with other analysis results. It involves the steps of hydrolysis, separation, detection and quantification. Hydrolysis of protein can be carried out using acid hydrolysis (6 M hydrochloric acid), alkaline

hydrolysis (4 M potassium, sodium or barium hydroxide) or enzymatic hydrolysis. Heating at approximately 110°C is normally applied for acid or alkaline hydrolysis for various periods ranging from 18 to 70 hrs. Enzymatic hydrolysis is rarely used for gluten proteins because the high proportion of proline prevents cleavage at adjacent residues (Koehler, 2003). Amino acids from protein hydrolysates are generally separated on ion exchange or RP-HPLC columns with derivatisation used to facilitate detection.

3.7 Summary of the current knowledge on wheat gluten protein

Wheat gluten protein is a complex system containing many polypeptides of gliadin and glutenins and stabilised through different bonds including disulfide, hydrogen and hydrophobic links as well as van der Waals interactions. The diversity of protein types and bonding gives gluten protein the unique functional properties, prompting the interest of cereal chemists over a long period.

Various studies have investigated the composition and structure of gluten proteins. The classification of the proteins has been based on solubility, electrophoretic mobility and amino acid sequences. Numerous methods and techniques have been developed to fractionate and identify protein types in relation to the quality of the various products made from wheat. Although these studies have been carried out over a number of decades, research continues in order to identify and characterise all of the many proteins present in gluten. Recently, many studies have concentrated on the isolation and identification of lipid-binding proteins in wheat. Much remains to be done if the interactions of gluten and lipids are to be understood.

Chapter 4

Background and literature review: Wheat lipids – classification, extraction and identification

The purpose of this chapter is to provide background and literature review on wheat lipids. The areas covered are the classification of wheat lipids, the methods of extraction, lipid identification and determination in wheat products and the distribution of lipids in the wheat kernel, flour and gluten.

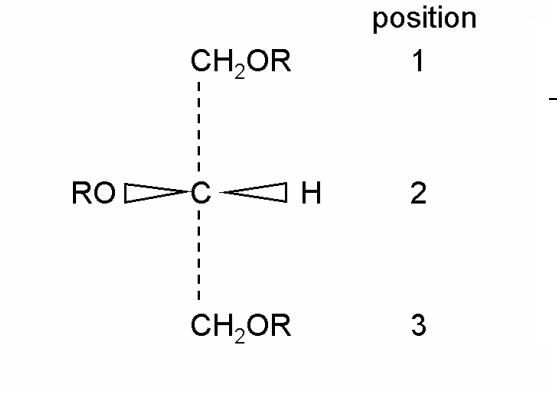
4.1 Structure and classification of wheat lipid

Lipid is a minor component of the wheat grain, and is primarily found in the germ, although small amounts are distributed throughout the kernel in membranes, organelles, and membrane-bound oil droplets. The composition of lipid, in wheat and in cereals generally, is very complex and diverse. The individual components can be classified as being either simple or complex lipids, based on their structure (Day, 2004). Simple lipids, which are composed of fatty acids and an alcohol component (most commonly glycerol), include acylglycerol, ether acylglycerol, sterols and their esters. Complex lipids, with more than two components in their structure, include phospholipids, glycolipids and sphingolipids. Based on the solubility of lipid in various solvents, wheat lipids can be separated into three broad classes using column chromatography on silicic acid; these are non-polar lipids in the first elution with chloroform, glycolipids in the next with acetone and finally phospholipids with methanol (Chung and Ohm, 2000). The glycolipids and phospholipids are generally regarded as the polar lipids.

4.1.1 Non-polar lipids

The non-polar lipids consist of sterol esters, triacylglycerols (TAG), diacylglycerols (DAG), monoacylglycerols (MAG) and free fatty acids. The mixture of any or all of TAG, DAG and MAG is termed as acylglycerols as they only vary in the number and position of acyl groups in their structure (Figure 4.1). In wheat lipids the predominant fatty acids, in order of abundance, are linoleate (18:2, n-6); palmitate (16:0); oleate (18:1, n-9); linolenate (18:3, n-3) and stearate (18:0) reflecting a relatively high

proportion of unsaturated fatty acids. TAG exist in spherisomes (oil droplets) bound by a monolayer membrane. The highest TAG levels occur in aleurone and scutellum tissues, but there are also substantial quantities in the cereal embryo and in the starchy endosperm. DAG are intermediates in the biosynthesis of TAG, glycosyldiglycerols and phosphoglycerols, although they may also be formed by lipolysis of TAG. MAG are generally associated with partly degraded TAG and are not present in significant amounts in the mature tissues of wheat kernels (Morrison, 1983).



Lipid	R at three positions
TAG	3 × acyl
DAG	1 × H, 2 × acyl
MAG	2 × H, 1 × acyl

Figure 4.1 The general structure of an acylglycerol molecule

4.1.2 Glycolipids

Glycolipids are compounds containing one or more monosaccharide residues bound covalently by a glycosidic linkage to a lipid component such as acylglycerol, ceramide and prenol. Glycoglycerolipids are glycolipids containing one or more carbohydrate unit, and these are the main components of the glycolipids in the starchy endosperm and whole grain of wheat. Monogalactosyl diglyceride (MGDG) and digalactosyl diglyceride (DGDG), which have one or two galactosyl derivatives, respectively, in their structure (Figure 4.2), are the predominant glycoglycerolipids in the endosperm. Small amounts of monogalactosyl monoglyceride (MGMG) and digalactosyl monoglyceride (DGMG) are also found in the endosperm of mature wheat when there is little evidence of lipase or lipolytic acyl hydrolase activity (Morrison, 1983).

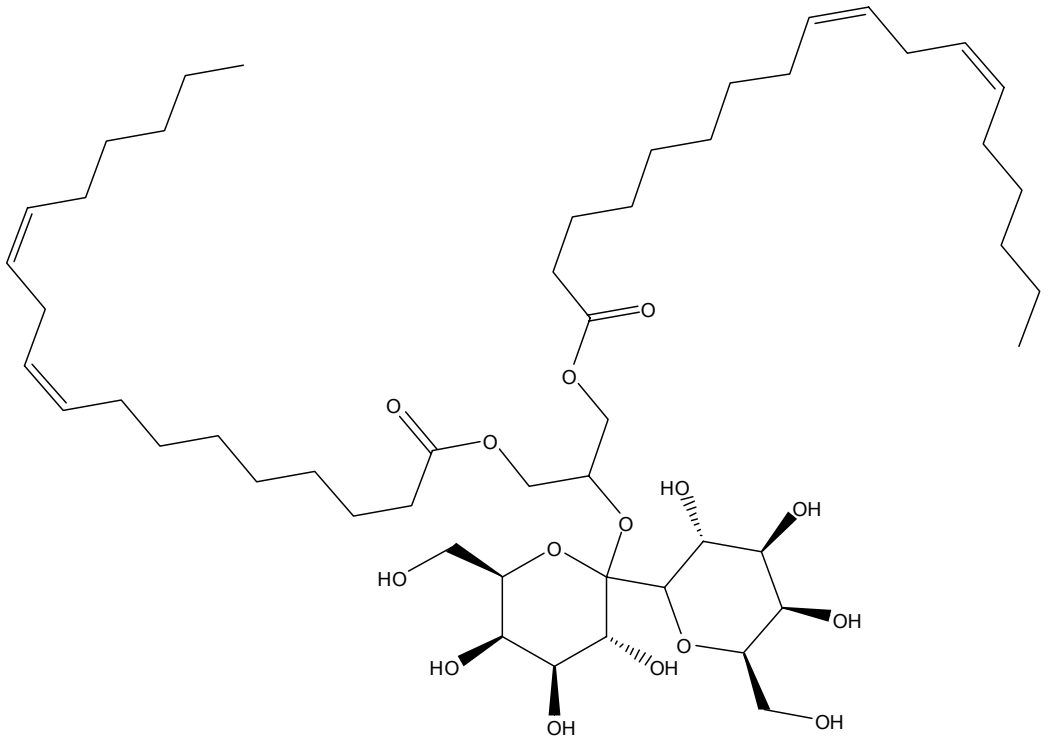


Figure 4.2 Structure of digalactosyl 1,3-dilinoleoyl glycerol in wheat

4.1.3 Phospholipids

Phospholipids are defined as those containing phosphoric acid as mono- or diester in their structure (Figure 4.3). The major phospholipids in wheat are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI). Minor phospholipids include phosphatidylglycerol (PG), phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE) and diphosphatidylglycerol (DPG). They are classified as membrane lipids and are reported to interact with proteins during dough development (Morrison, 1983).

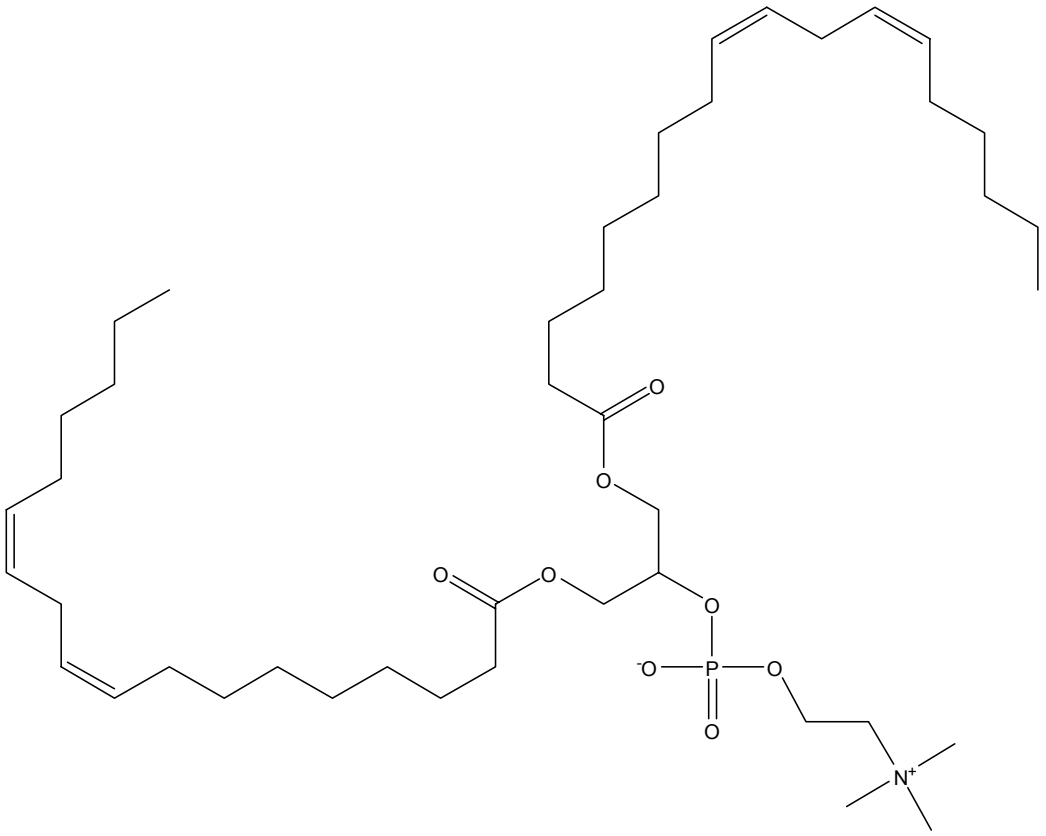


Figure 4.3 Structure of phosphatidylcholine, based on linoleic acid.

4.2 Wheat lipid extractability

Wheat lipids can be categorised as being free or bound, based on their extractability with particular solvents. Free lipids are the portions readily extracted with non-polar solvents including petroleum ether, hexane and diethylether, while bound lipids are extracted with solvents of greater polarity – generally an alcohol or a mixture of an alcohol and water (Cornell and Hoveling, 1998b; Chung and Ohm, 2000). The total of free and bound lipids is also referred to as the total non-starch lipids. The reason for this is that the conditions used to measure free and bound components do not extract lipids located within the starch granules. It is noted that some lipids on the surface of starch granules can be extracted as free or bound lipids. The total non-starch lipids can be measured directly by polar solvent extraction at room temperature without the use of a prior free-lipid extraction step (Chung and Ohm, 2000). In wheat, free and bound lipids may include non-polar lipids, glycolipids and phospholipids.

Wheat lipids also exist in a starch-bound form known as starch lipids. They are the most difficult components to extract since they are present inside the starch granule. Their extraction requires hot aqueous alcohol under conditions that control swelling of the starch granules but allow solubilisation of the lipids. The most effective solvents for starch lipid extraction contain either *n*-propanol or isopropanol with water (3:1, v/v) under nitrogen at 100°C while combinations of methanol or butanol with water are reasonably efficient for the extraction (Morrison and Coventry, 1985).

4.3 Wheat lipid extraction and determination

4.3.1 The acid hydrolysis method

The standard method of acid hydrolysis (AACC, 1995a) is suitable for determining the total lipid in finely ground materials. The weight of hydrolysate lipid is close to the true lipid weight from products rich in TAG but, for some samples, the result may be impacted by the exclusion of polar moieties from glycolipids and phospholipids or the inclusion of lipophilic artifacts (Morrison, 1978). The sensitivity of the acid hydrolysis method is limited through the selection of a suitable sample size in order to obtain a reproducible amount of lipid for weighing. An approach to increasing sensitivity and to eliminating the problem of non-lipid artefacts involves converting hydrolysate lipids into fatty acid methyl esters (FAME) for quantitative gas liquid chromatography (GLC), using heptadecanoate (17:0) as an internal standard (Morrison et al., 1980).

In the acid hydrolysis method, lipids are hydrolysed before extraction. The lipids obtained in the lipid extract are mainly fatty acids, so this lipid extract cannot be used for identifying the lipid classes. On the other hand, a lipid extract from solvent extraction can be used for further analysis of lipid classes; however, the efficiency of lipid extraction from a sample is dependent on the solvent and the procedure. The sufficiency of the extraction technique or the completeness of solvent extraction can be assessed by comparing the results of solvent extraction and the acid hydrolysis method. It has been suggested that the results are not strictly comparable, as the amount of lipid determined by the acid hydrolysis method is the total of the fatty acids (Cornell and

Hoveling, 1998b), but the approach remains useful as the basis of assessment of extraction procedures.

4.3.2 Solvent extraction methods

Wheat lipids can be extracted under cold or hot conditions using organic solvents. In addition, various types of apparatus may be employed, including the Goldfisch extractor, Soxhlet apparatus and mechanical mixers. In all cases, the lipid content is determined using gravimetric analysis. The amount and types of lipid extracted depend on the extraction and purification procedure, such as type of solvent, extraction conditions and apparatus, as well as sample attributes of variety, particle size and moisture content. Therefore, it is difficult to compare lipid content or compositional data from various reports (Chung and Ohm, 2000).

Many single and mixed solvent extraction methods have been used to evaluate the extraction of wheat lipid (Morrison, 1978). Solvents of low polarity, including hexane or petroleum ether, are unable to extract all of the lipids from flour as they can only extract those which are in the free form (Morrison, 1976). The efficiency of the extraction differs between solvents, with chloroform able to extract more lipids from flour than petroleum ether (Meredith et al., 1960; McCormack et al., 1991). More polar solvents, including ethanol, *n*-butanol saturated with water (WSB) or chloroform / methanol (2:1, v/v), provide a better lipid extraction for flour or dough but the extent achieved differs among these solvents (Cornell and Hoveling, 1998b).

WSB is considered to be the most effective solvent for lipid extraction of flour, as it gives results close to those obtained from the acid-hydrolysis method (Mecham and Mohammed, 1955). Colborne and Laidman (1975) reported that WSB was also the most effective solvent for the extraction of total phospholipids from whole-wheat grains. Although the presence of phospholipase D and transphosphatidylase in bran could affect the level of N-acyl PE during extraction, this can be overcome by denaturing the tissue by heat before extraction with WSB. However, some non-lipid material was found to be extracted with this solvent (Cornell and Hoveling, 1998b). The boiling point of *n*-butanol is relatively high, so the removal of solvent after extraction is a problem, requiring further extraction with a low-boiling-point solvent.

A mixture of chloroform/methanol (2:1, v/v) is able to extract lipid from wheat flour (Pomeranz and Chung, 1965) and dough (Tsen et al., 1962) in which strong lipid-binding occurs. Ethanol is efficient in extracting lipid from flour, as the amount of lipid extracted using ethanol is higher than other single solvents including benzene, petroleum ether and chloroform (Meredith et al., 1960) or the mixture of chloroform/methanol (Tsen et al., 1962).

Selective extraction with WSB performed at 20°C and subsequently at 90 – 100°C has been used to extract non-starch and starch lipids from wheat flour, respectively (Morrison et al., 1975). Chung and Tsen (1975a) selectively extracted free lipid from flour and flour fractions using petroleum ether and then extracted bound lipid from the petroleum ether extracted material using WSB. Recently, selective extraction has been developed to firstly extract free lipid with petroleum ether and finally bound lipid with an acid-hydrolysis method (Ruibal-Mendieta et al., 2002).

4.4 Lipid class identification and quantification

A total of 23 lipid classes has been identified in wheat lipid extracted using WSB; these include steryl esters (7.5%), TAG (20.8%), DAG (12.2%) and free fatty acids (7.0%) as the major free lipids, DGDG (13.5%), MGDG (4.9%) as the major glycolipids and LPC (7.1%), PC (5.8%) and N-acyl PE (2.9%) as the major phospholipids (MacMurray and Morrison, 1970).

Many methods have been developed to successfully separate and identify the lipid classes of wheat lipids using thin-layer chromatography (TLC) (Pomeranz and Chung, 1965; Clayton et al., 1970) and high-performance liquid chromatography (HPLC) in conjunction with an evaporative light-scattering detector (ELSD) (Conforti et al., 1993; Néron et al., 2004). Other methods have been used to determine the content of free glycolipids in flour using scanning spectrophotometry (Ohm and Chung, 2000) or the content of fatty acids using transesterification of fatty acids and gas chromatography (Morrison et al., 1980).

4.4.1 Thin layer chromatography

TLC has been commonly used for separating lipid classes of wheat samples. The separation of lipid is based on the difference in the affinity of components towards a stationary and a mobile phase (Shantha and Napolitano, 1998). The most popular stationary phase materials for lipid separation are silica gel, alumina and kieselguhr. The TLC plate made with a thin layer of silica gel is activated before being used. After spotting a small amount of lipid material, the plate is developed in paper-lined tanks with a mixed solvent system functioning as the mobile phase. The separated lipid spots can be visualised temporarily as brown spots by exposure to iodine vapour or under ultraviolet (UV) light after spraying with a fluorescent agent, or permanently as dark spots by charring above 160°C following spraying with sulfuric acid (10–50% in ethanol, v/v) (Day, 2004). Temporarily visualised spots permit the recovery of the components of the lipid classes on the silica gel for preparative or analytical purposes. Acid-charred spots can be scanned with a densitometer for quantitation purposes. However, different classes of lipid give differential responses due to the characteristics of the components and degree of unsaturation (Cornell and Hoveling, 1998b). Bitman and Wood (1982) found that a mixture of 10% copper sulfate in 8% phosphoric acid combined with a temperature-program heating (30–180°C at 10°C/min) could improve reproducibility as well as evenness of response for different lipid classes.

The separation of lipid classes on a TLC plate depends on the solvent system used. The most useful solvents for ascending development of lipid spots were chloroform, a mixture of petroleum ether/diethyl ether/acetic acid (80:20:1) or a mixture of chloroform/methanol/water (65:25:4) (Pomeranz and Chung, 1965). Clayton et al. (1970) studied the effect of solvent systems on the separation of lipid and it was shown that a mixture of diethyl ether/benzene/ethanol/acetic acid (40:50:2:0.2) is suitable for the separation of neutral lipids; a mixture of chloroform/acetone/water (30:60:2) is good for the polar glycolipid separation; and a mixture of chloroform/methanol/ammonia (30%, w/v)/water (65:30:5:2.5) is able to resolve most polar glycolipids and some phospholipids. Other phospholipids, including LPC, PS and PI, were not well separated with the solvent system used by Clayton et al. (1970); however, enhanced separation could be achieved with a mixture of chloroform/methanol/acetic acid/water (65:25:8:4) (Morrison, 1968).

4.4.2 Gas liquid chromatography

GLC is a useful technique for quantitative determination of the fatty acid composition of lipids. Fatty acids must be treated with either a saponification-methylation method or a transesterification method to increase their volatility before application to GLC. Transesterification, based on the preparation of FAME, is a popular method as it is rapid and low-cost, and because it causes minimal degradation of samples (Cornell and Hoveling, 1998b). Morrison et al. (1980) have combined TLC with GLC in the quantitation of lipid classes in cereal extracts. After separating lipid classes on TLC, the material from each spot was collected, recovered and applied to the GLC column. The quantity of FAME was calculated and then converted into the weight of original lipid using the FAME factor. Although this method could be used to quantify some lipid classes of cereal products, a misidentification could occur as some lipids could not be separated on TLC.

4.4.3 High performance liquid chromatography

The concept of lipid separation by HPLC is similar to that of TLC. However, the resolving power of HPLC is greater and the separation of lipids by HPLC is not currently rivalled by other methods. Similar to any technique in liquid chromatography, the process of HPLC involves the partitioning of components between mobile phase and stationary liquid/solid phase in a column. Columns can be packed with different types of materials including silica gel or alumina, octadecylsilyl linked to a silanol surface (C-18) or a substituent group bonded to a silanol support. There are several modes of separation in HPLC and these are based on different molecular interactions between the solute and the mobile and stationary phases. Reversed-phase and normal-phase liquid partition chromatography are commonly applied for lipid analysis. Ion exchange, gel permeation and chiral-phase chromatography are rarely used for lipid separation, although gel-permeation chromatography has proved to be of value for the separation of polymerised lipids, lipoprotein complexes and apolipoproteins (Christie, 1987).

The availability of a sensitive and suitable detector has been a barrier for extending the application of HPLC in lipid analysis (Christie, 1987). As lipids lack chromophores to facilitate spectrophotometric detection, wavelengths in the UV range from 200 to 210

nm have been used. There are a number of disadvantages to this approach because most lipids exhibit a weak absorbance whilst many solvents used for their chromatography, including chloroform, absorb strongly in this range. Furthermore, the presence of an antioxidant or plasticiser in the solvent could disturb the base-line and give high background readings.

A refractive index detector and reversed-phase HPLC (RP-HPLC) has been used to separate and quantify MGDG and DGDG in the polar lipid fractions of wheat flour. The detection limit of this type of detector with interferometric optics was 0.25 μg for both DGDG and MGDG (Tweeten and Wetzel, 1981). Other limitations on the use of such detectors in the direct quantitative analysis of lipid are that they can only be used under isocratic elution conditions, and that they are sensitive to changes in temperature and flow rate of solvent (Christie, 1987).

The development of ELSD has overcome the limitations of UV and refractive index detectors as there is no particular wavelength required for the light source of the detector and it is not affected by changes in ambient temperature. During ELSD operation, the solvent of the column eluate is evaporated in a stream of nitrogen in a heating chamber whilst the solute is nebulised and passed through a light beam in the form of minute droplets, allowing detection by the reflection or refraction of the light (Charlesworth, 1978). ELSD provides excellent results under gradient elution; however, there are some limitations of this detector including the choice of solvents and large amounts of gas required for nebulisation. Solvents used for this detector must be sufficiently evaporated in the heating chamber. In addition, the presence of salts or ionic materials can reduce the sensitivity of the detector (Christie, 1987), because these materials can affect the uniformity of the solute droplets in the heating chamber. For the same reason, the response of peak area to lipid mass is non-linear when the concentration of lipid is too high or low. It has been reported that the peak area of ELSD has a linear response with the mass of most lipids in the range of 10 to 200 μg (Moreau, 1994).

HPLC in conjunction with ELSD has been used to separate and identify lipids of unbleached soft red winter wheat flour. The separation of the major starch and non-starch lipids was achieved in 60 min using a Lichrosorb Si-60 silica cartridge system

and ternary gradient system; the peak areas were linear with the mass of various phospholipids and glycolipids including PE, LPE, LPC, MGDG and DGDG in the range of 10-200 μg (Conforti et al., 1993). Papantoniou et al. (2001) have scaled up this method for the isolation of polar lipids from wheat flour in relatively large quantities. More recently, Néron et al. (2004) developed a method for separation and quantification of phospholipids in wheat flour during dough mixing in the presence of phospholipase with a shorter running time. The peak areas for a range of lipids showed linear responses from 0.01 to 0.09 μmoles (Néron et al., 2004). Using gradient elution, Christie and Urwin (1995) were able to separate a wide range of lipids derived from plant tissue, which included non-polar lipids, glycolipids and phospholipids. This method has been modified for analysing phospholipids in milk (Fagan et al., 2004) and various lipid classes from seawater (Nordbäck et al., 1998).

4.5 Distribution of lipids in wheat kernel, flour and gluten

Wheat-flour lipid contains approximately 70% of non-starch lipids and 30% of starch lipids (Morrison et al., 1975). Non-starch lipids consist of non-polar lipids (59–63 %), glycolipids (22–27%) and phospholipids (13–16%). Steryl esters, TAG and all galactosylglycerols and phosphoglycerols are present only in non-starch lipids. Starch lipids consist of non-polar lipids (including free fatty acids, 6–9%), glycolipids (3–5%) and phospholipids (particularly LPC, 86–91%) (Morrison et al., 1975; Chung and Ohm, 2000).

Wheat lipids are not distributed evenly throughout the wheat kernel. The non-starch lipid content of different wheat kernel fractions is summarised in Table 4.1. Non-polar lipids are the predominant lipids in germ and aleurone, while high proportions of polar lipids (glycolipids and phospholipids) are present in the endosperm. The highest concentrations of TAG exist in the aleurone and germ, and the lowest in the endosperm. MGDG and DGDG are the major glycolipids found in the endosperm, while phospholipids are typically present in the germ and aleurone. The endosperm is rich in DGDG whereas the bran contains a high concentration of PC (Morrison, 1988; Chung and Ohm, 2000).

Table 4.1 Non-starch lipid content in wheat kernel fractions

	Lipid content (g/100 g of fraction, dry basis)				
	Pericarp	Germ	Aleurone	Aleurone-free endosperm	
				Tetraploid	Hexaploid
Total non-polar lipid	1.14	15.6–22.9	6.29–8.82	0.37–0.40	0.35–0.39
SE	0.17	0.43–0.57	0.11–0.32	0.01–0.02	0.02–0.05
TG	0.20	14.3–22.0	5.25–7.98	0.26–0.27	0.14–0.22
DG	0.46	0.10–0.44	0.08–0.32	0.04–0.05	0.04–0.06
MG	0.02	0.11–0.20	0.05–0.08	0.01–0.04	0.02–0.08
FFA	0.28	0.03–0.28	0.17–0.40	0.03–0.05	0.04–0.06
Total glycolipids	0.09	0.19–0.52	0.23–0.85	0.15–0.17	0.26–0.41
ASG	0.05	-	0.16–0.24	0.01–0.02	0.01–0.04
MGDG	-	-	0.05–0.09	0.02	0.03–0.08
MGMG	-	0.19–0.53	0.01–0.06	0.01	0.01–0.02
DGDG	-	-	0.04–0.21	0.11	0.15–0.28
DGMG	-	-	0.03–0.21	0.01	0.02
Total phospholipids	0.09	1.97–2.65	1.48–1.58	0.23–0.27	0.19–0.39
PE	-	0.36–0.38	0.13–0.15	-	-
PC	-	0.88–2.38	0.78–0.88	0.02	0.02–0.07
PI	-	0.26–1.10	0.25–0.27	-	-
LPE	-	0.01–0.19	-	-	-
LPC	-	0.08–0.36	0.06–0.21	0.09–0.10	0.04–0.10

Source: (Morrison, 1988).

During the maturation of wheat, lipids are formed and their distribution within the kernel changes. There is an increase in the amount of TAG, accompanied by a decrease in levels of MAG, DAG and free fatty acids. Large amounts of N-containing compounds are found in the fractionation of polar lipids from maturing wheat when

spraying with ninhydrin. These decrease or disappear whilst galactosylglycerols and PC increase substantially during maturation (Pomeranz and Chung, 1965).

The free lipid and non-polar lipid contents of wheat and wheat flour vary due to differences between wheat cultivars, growth locations and harvest time; these influences have been reviewed by Chung and Ohm (2000). The free lipid content of hard and soft wheat grown in Canada ranged from 72 to 134 mg per 10 g flour (dry basis), 61–115 mg for non-polar lipid, 4–11 mg for glycolipid, and 4–9 mg for phospholipid (Bekes et al., 1986). The variation of free-lipid content is larger in Canadian spring wheat than in the United States (US) hard winter wheat (Chung and Ohm, 2000). Soft winter wheat in the United Kingdom (UK) contained higher free-lipid content (195–244 mg per 10 g, dry basis) with a higher proportion of non-polar lipid content than hard winter wheat (186–210 mg per 10 g, dry basis) (Bell et al., 1987). Generally, UK hard spring wheat contained higher free-lipid content than the US hard winter wheat. In comparison, Australian wheats contained substantially less free-lipid and non-polar lipid but were higher in phospholipid, whilst the wheat flours contained similar levels of free lipid and non-polar lipid and were higher in polar lipids (Panozzo et al., 1990; McCormack et al., 1991).

The compositions of free and bound lipids extracted from flour are quite different. Approximately two-thirds of free lipids are non-polar lipid while two-thirds of bound lipids are polar lipid. Free polar lipid is richer in glycolipid than phospholipid, whereas bound polar lipid is slightly richer in phospholipid (Chung, 1986).

The lipid content of gluten prepared at a laboratory scale ranges from 5 to 10% (Dill, 1925; Olcott and Mecham, 1947; Ponte et al., 1967). Only small amounts of this lipid can be extracted with non-polar solvent such as ether (Olcott and Mecham, 1947; Hoseney et al., 1970a). In contrast to flour that generally contains more non-polar lipid than polar lipid, gluten contains slightly more polar lipid than non-polar lipid (Chung, 1986). The composition of free and bound lipid in gluten is quite different to flour. While free lipid in flour contains both non-polar lipid and polar lipid, free lipid in gluten has only non-polar lipid components (Hoseney et al., 1970a). Bound polar lipid in flour is rich in phospholipid, whereas in gluten, it is rich in non-polar lipid and glycolipid (Hoseney et al., 1970a). Differences in the distribution of lipids in flour and gluten

could be attributed to the association with proteins during dough formation and gluten preparation. This area is reviewed and discussed in Chapter 5.

4.6 Summary of the current review on wheat lipid

Composition of wheat lipid is very complex. Broadly, it consists of three main classes: non-polar lipids, glycolipids and phospholipids. Many methods have been successfully developed to extract and determine amount of lipids in wheat kernel, flour and gluten. Recently, HPLC in conjunction with ELSD could analyse a wide range of lipids in a reasonable running time. The determination of lipid indicated that wheat lipids were not distributed evenly throughout the kernels. The proportion of non-polar lipids, glycolipids and phospholipids varied, depending on maturation and varieties of wheat. These lipids occur in flour and gluten as free and bound lipids. The composition of the free and bound lipids in flour is different to that in gluten although gluten is made from flour through the starch-removal process. The differences in lipid distribution in flour and gluten could be due to the association of wheat lipids and proteins during gluten preparation.

Chapter 5

Background and literature review: Interactions of protein and lipid in dough and gluten

The purpose of this chapter is to provide background and to review the relevant scientific literature on the interactions of protein and lipid in dough and gluten. The areas covered are the evidence of lipid binding in dough and gluten, the effects of protein and lipid interactions on the quality of bakery products, a review of current studies of these interactions and a summary of the proposed mechanisms.

5.1 Lipid binding in dough and gluten

It has been widely recognised that lipid binding occurs during dough development and gluten preparation (Frazier, 1983; Chung, 1986; Carr et al., 1992). This is indicated by a decreasing level of free lipid with a corresponding increase in the amount of bound lipid during dough mixing. Approximately 70% of flour lipid, originally present in the free form, became bound during dough mixing (Olcott and Mecham, 1947). The association of lipids is particularly with gluten proteins, although albumin and globulin proteins have been implicated to a certain extent (Olcott and Mecham, 1947; Ponte et al., 1967; Hoseney et al., 1970a; Chung and Tsen, 1975c; Frazier et al., 1981).

Lipid binding can occur immediately after the addition of water to flour (Olcott and Mecham, 1947), even without work input (Davies et al., 1969). Increasing the moisture content of flour results in increasing levels of binding and this reaches a maximum at a moisture content of 45% (Davies et al., 1969). The binding of lipid primarily occurs during the first stage of mixing and continues until the optimum mixing time (Chung and Tsen, 1975a; Chung, 1986). It has been reported that one half to two thirds of free lipid becomes bound by the point of optimum mixing (Chung, 1986). In this phenomenon, free non-polar lipid (other than diacylglycerols) decreases with increasing mixing time whilst polar lipid that has initially been free becomes completely bound at the first stage of mixing (Chung and Tsen, 1975a). When oil was added to a dough formulation, the amount of the added oil (which was in the free form) was found to

decline as the work input was increased (Frazier et al., 1981). It has also been shown that the level of lipid binding can be markedly affected by the presence of oxygen in the dough-mixing chamber (Daniels et al., 1969). Prolonged overmixing of dough in a normal atmosphere can slightly reduce the proportion of bound lipid (Pomeranz et al., 1968); whereas in the absence of oxygen, the level of lipid binding can continue to increase with high levels of work input (Daniels et al., 1971).

The various lipid classes appear to compete with each other when they are associating with proteins. It has been reported that increased levels of non-polar lipid are able to reduce slightly the binding of polar lipid to protein (Pomeranz et al., 1968). However, unsaturated free fatty acids, that are classified as non-polar lipid, have been found to increase the extent of lipid binding (Bell et al., 1979).

5.2 The effect of interactions of protein with lipid on the quality of bakery products

Wheat lipids play an important role in forming the viscoelastic properties of dough and contributing to loaf volume. Removal of lipid from flour tends to strengthen the rheological properties of the resulting dough, but dough texture is less smooth. Nevertheless, the dough becomes smoother during proofing. Removal of non-starch lipids from flour has been reported to increase loaf volume. Reconstitution of the extracted lipids into the flour initially decreases loaf volume, but the volume increases when more than 50% of the extracted lipids is added (MacRitchie, 1983).

It has been reported that the loaf volume of bread is strongly related to the non-starch lipid content and to the level of polar lipids (McCormack et al., 1991; MacRitchie and Gras, 1973). In a series of definitive experiments (MacRitchie and Gras, 1973; MacRitchie, 1983) polar lipids had a positive effect on the loaf volume while non-polar lipids were detrimental. Increasing the proportion of the non-polar fraction produced a progressive decrease in loaf volume, while increasing the polar fraction tended to increase the loaf volume. However, there was an initial reduction of volume when the level of polar lipid was less than 0.3%. Increasing the non-polar lipid fraction increased crumb firmness whilst the polar lipid tended to form a finer and more uniform crumb. Of the non-polar fraction, MAG, DAG and TAG had no significant effect on loaf

volume but free fatty acids caused a depression in loaf volume. The presence of some free fatty acids in the polar lipid fraction could explain the initial reduction of volume when the polar lipid fraction was reconstituted into flour. Of the polar fraction, glycolipids were most effective in increasing loaf volume both in the presence and absence of shortening. Phospholipids had no effect in the absence of shortening but significantly increased loaf volume in their presence (MacRitchie, 1983).

For the production of cookies, glycolipids and phospholipids in the polar lipid fraction of flour were found to be functionally beneficial (Cole et al., 1960; Clements and Donelson, 1981). It was also found that polar and non-polar lipid fractions alone are only partially effective in improving defatted flour (Kissell et al., 1971), in which the polar lipid fraction was of greatest benefit (Papantoniou et al., 2003). However, both were required to fully restore the quality of biscuits and the rheological properties of dough (Kissell et al., 1971; Papantoniou et al., 2004).

In cake manufacture, the free lipid fraction of flour was found not to be a factor governing baking potential (Spies and Kirleis, 1978). However, there was some evidence indicating the impact of particular lipid fractions on the quality of cakes. In a reconstitution study, the level of the polar-lipid fraction of flour correlated with cake volume (Kaldy et al., 1993). The MGDG and DGDG fractions from the free flour lipid could improve cake expansion, whilst the TAG fraction had no effect (Takeda, 1994).

Many studies on baked products have investigated the role of lipid in forming and stabilising gas-cell structure (MacRitchie and Gras, 1973; MacRitchie, 1983). The various lipid components of flour have different surface activities. Glycolipids and phospholipids, in association with protein, act as foam stabilisers while non-polar lipids including free fatty acids act as foam de-stabilisers (MacRitchie, 1983). Lipid and protein from flour are found to co-exist in interfacial films and contribute to their stability (Gan et al., 1995; Marion et al., 1998)

The stabilisation of gas cells in bread dough development was found to involve the distribution of fat crystals on the gas cell surface (Brooker, 1996), and the association of phosphoglycerides with gliadin in the network (Li et al., 2004). Upon addition of a small amount of crystalline fat into bread dough, the fat firstly developed a crystal-water

interface and was then adsorbed onto the gas-liquid interface of bubbles (Brooker, 1996). The number of adsorbed fat crystals increased progressively as the bubbles expanded during proofing. During baking, crystals melt and the fat-liquid interface became incorporated into the bubble surface. As a result, the addition of crystalline fat could stabilise the gas cell during proofing and the baking process, thereby contributing to loaf volume and crumb structure.

5.3 Lipid in protein fractions

Many studies on the interaction of lipid and protein have investigated the distribution of lipid in the protein fractions of flour and gluten (Olcott and Mecham, 1947; Ponte et al., 1967; Hoseney et al., 1970a; Chung and Tsen, 1975a). This approach has been used to identify the interactions of protein and lipid (Table 5.1). However, the results of the various studies are not consistent because the lipid distribution between the gluten fractions has been found to depend on the fractionation method used (Chung, 1986). The use of dilute acid with dough or gluten yields lipid primarily in the HMW glutenin fractions, while fractionation based on organic solvent or detergent yields lipid not only in the glutenin but also in lower molecular weight fractions (Olcott and Mecham, 1947; Ponte et al., 1967; Chung, 1986; Carr et al., 1992).

Early studies found that gluten was fractionated into gliadin and glutenin using pH precipitation; more than 80% of gluten lipid was reported to be associated with glutenin and very little was found in the gliadin fraction. Phospholipids were reported to be bound preferentially during dough development. The presence of “lipoglutenin” in wheat gluten was suggested (Olcott and Mecham, 1947).

Another study, using 70% ethanol for the fractionation of glutenin and gliadin from a 0.05 N acetic acid soluble gluten fraction, found that gliadin proteins contained significantly more lipid than did glutenin protein (Ponte et al., 1967). The gliadin fraction consisted of considerable amounts of polar lipids, while the glutenin was richer in TAG; this report is in contrast to the results of Olcott and Mecham (1947).

Table 5.1 Protein and lipid interactions identified from fractionation studies

Fractionation method	Materials	Protein-lipid interactions	Reference
pH precipitation	Gluten: Acetic acid soluble	- Lipid in glutenin > in gliadin	(Olcott and Mecham, 1947)
Ammonium sulfate precipitation	Flour plus oil: Acetic acid soluble	- Added lipid in glutenin > in gliadin - High level of lipid in acetic acid soluble fraction	(Frazier et al., 1981)
70% ethanol	Flour plus oil: Acetic acid soluble	- Both glutenin and gliadin associated with added lipid	(Frazier et al., 1981)
70% ethanol	Gluten: Acetic acid soluble	- Lipid in glutenin < in gliadin - Gliadin associated with polar lipid; glutenin with TAG	(Ponte et al., 1967)
70% ethanol	Gluten: 0.005 N lactic acid soluble	- Lipid in glutenin < in gliadin - Gliadin: free lipid > bound lipid (glycolipids) - Glutenin: free lipid < bound lipid (glycolipids) - Both glutenin and gliadin associated with glycolipids	(Hoseney et al., 1970a)
Gel filtration Bio-Gel P-150	Flour: Acetic acid soluble	- Glutenin associated with non-polar lipids and glycolipids - Gliadin and albumin with only non-polar lipids	(Chung and Tsen, 1975c)
Gel filtration Sephadex G-200	Alcohol soluble and insoluble fractions	- Glutenin associated with non-polar lipids and polar lipid (mostly phospholipids) - HMW-gliadin and S protein associated with glycolipids	(Bushuk, 1985)
0.05 M acetic acid	Flour	- Acetic acid soluble fraction: non-polar lipids and glycolipids - Insoluble protein and starch fractions: polar lipids	(Chung and Tsen, 1975a)

Similar results to those of Ponte et al. (1967) were obtained by Hoseney et al. (1970a), who dissolved gluten with 0.005 N lactic acid and then fractionated it into glutenin and

gliadin using 70% ethanol. The latter fraction was relatively high in free lipid whilst more bound than free lipid was present in the glutenin. The bound lipid in the gliadin consisted of only polar lipid components, primarily glycolipids (MGDG and DGDG). Glycolipids were found to associate with both the gliadin and glutenin fractions. It has been suggested that a gliadin-glycolipid-glutenin complex exists in wheat gluten (Hoseney et al., 1970a).

Chung and Tsen (1975c) studied the lipid distribution in four protein fractions obtained when acetic acid soluble proteins are separated on a Bio-Gel P-150 column. The results are in contrast to the earlier findings of Ponte et al. (1967) based on ethanol precipitation. In the study of Ponte et al. (1967), polar lipids were found to associate with the gliadin fraction. Whereas, the findings from Chung and Tsen (1975c) indicate that polar lipids, especially glycolipids, are associated with glutenin and also non-polar lipids predominate in gliadin and albumin fractions.

In another study, Chung and Tsen (1975a) fractionated flour into acetic acid soluble, gelatinous, starch-lipid-protein and starch fractions using 0.05 M acetic acid solution. They found that the gelatinous fraction was richest in both free and bound lipid. Non-polar lipids were preferentially bound to acid-soluble protein and polar lipids were possibly associated with acid-insoluble protein and starch. Fatty acid and glycolipids were particularly bound to both acetic acid soluble and starch-lipid-protein fractions. A similar finding was reported by Frazier et al. (1981), indicating that the acetic acid soluble proteins from dough could be responsible for the binding of lipid during dough mixing under nitrogen and involved in oxidative interactions with dough lipids. However, the addition of surfactant into flour appeared to decrease protein extractability and the amount of lipids bound to the acetic acid soluble fraction (Chung and Tsen, 1975b).

Labelled lipid (olive oil) was added into flour for studying the distribution of lipid in the protein fraction of dough using two different fractionation methods (Frazier et al, 1981). In the first, the separation of glutenin and gliadin in the acetic acid soluble fraction using 70% ethanol indicated that both glutenin and gliadin are responsible for lipid binding during dough formation. Glutenin could be an important fraction in determining the pattern of lipid binding. In the second method, ammonium sulfate precipitation of

the acetic soluble fraction showed that precipitated glutenin fraction contains the highest proportion of labelled lipids.

From gel filtration on Sephadex G-200, glycolipids, particularly DGDG, were found to have an association with HMW gliadin (65−80 kDa) and LMW proteins (S protein) in fraction I of the gliadin (alcohol soluble) fraction (Bushuk, 1985). Both non-polar and polar lipids were associated with the glutenin (alcohol insoluble) fraction, in which the major component of the polar lipids was phospholipid. Fraction I of the glutenin fraction containing the intermediate molecular weight protein (45−60 kDa) was particularly found to associate with lipids (Bushuk, 1985).

The inconsistencies in the results of the various studies could be due to the fact that these have focused on the distribution of lipid in the protein fractions. On the other hand, the gluten matrix is a complex network of protein involving different types of bonding and interactions (Pomeranz, 1987; Bushuk, 1998; Wrigley et al., 2006). The chemicals used for fractionation in these studies, having different chemical properties; might have differing affects on the gluten matrix, leading to contrasting conclusions. Therefore, the distribution of both lipid and protein components in gluten fractions as well as the effect on bonding in the matrix under chemical treatments should be considered in interpreting the experimental results in order to provide a more complete understanding of the interactions.

5.4 The investigation of protein-lipid interactions with physical approaches

A number of physical techniques have been used to study the chemistry of lipid binding. They have consistently indicated close protein-lipid interactions in the many reports on dough. Relatively few researchers have specifically considered the interactions occurring during the preparation of gluten. In an aqueous medium, the lipid and protein components of a gluten gel exhibit self-association into colloidal aggregates and no evidence has been found for molecular lipid-protein interactions in the formation of this system (Larsson, 1985). Phosphorus magnetic resonance spectroscopy and freeze-fracture electron microscopy of wheat gluten have shown that lipids are associated only with the granular protein network and typically organised in vesicles. In these, polar lipids occurred in a lamellar liquid crystalline phase of small vesicles while non-polar

lipids existed in the smooth vesicles (Marion et al., 1987). Evidence indicates that lipids are retained within the gluten in a fairly non-specific way (Marion et al., 1987; Carr et al., 1992).

On the other hand, infrared and nuclear magnetic spectroscopy has provided evidence for the presence of chemical interactions including polar and hydrophobic bonds between polar lipids and certain components in dough (Wehrli and Pomeranz, 1970). This work found the occurrence of hydrogen bonds between glycolipids, gelatinised starch, and gluten components and van der Waals bonds between glycolipids and gluten components. Hence, it has been suggested that the gluten network retains lipid through a combination of forces, involving physical entrapment as well as polar or ionic bonding between protein and the surface of lipid phase (Carr et al., 1992).

Polar lipids tend to assist in the formation of a lipid monolayer at the gas-lipid interface of dough (Larsson, 1985). Recent work using confocal scanning laser microscopy has demonstrated that the association of polar lipids, primarily phospholipids, with gliadin has occurred in the gluten network and on the gas cell surface of bread dough (Li et al., 2004). During proofing of dough, glutenin was found only in the bulk of dough whilst gliadin was found in the bulk of dough as well as on the gas-liquid interface. Polar lipids also interacted with many particles in the proofed dough, which were believed to be starch granules and yeast cells. DGDG significantly contributed in stabilising the lamellar liquid-crystalline phase from dough, while free fatty acids had a negative effect on the liquid phase behaviour (Larsson, 1985). These findings correlate with the effect of polar lipids and free fatty acids on the loaf volume and crumb texture.

Competition between polar lipids and wheat proteins is observed on the gas cell interface in dough. Monolayer-forming lipids (PE, LPC and MGDG) incorporate more effectively than bilayer-forming lipids (DGDG) into the protein film of the gas cell. Polar lipids are able to replace proteins at the gas-cell interface and interfere with the protein and protein interactions (Keller et al., 1997; Paternotte et al., 1994). The abilities of wheat proteins to interact with monolayer lipids are related to their surface activities that have been found to increase in the order from albumin, globulin, glutenin to gliadin (Keller et al., 1997). Therefore, gliadin would be the most likely protein to appear on the gas cell interface and to interact strongly with lipids.

The lipid composition of wheat flour affects the behaviour of an aqueous extract of flour. It was observed that, after removal of lipids, an aqueous suspension from flour had higher foaming properties than did an extract from untreated flour (MacRitchie, 1983). The addition of fat to dough can improve the loaf volume; however, it also increases the surface tension of the aqueous phase. The improvement of loaf volume is claimed to be due to the arrangement of crystalline fat on the gas bubble surface (Brooker, 1996). The depletion of the surface activity of lipid material in the aqueous phase could be due to the ability of fat crystals to interact with flour lipids, allowing these lipids to occupy the gas cell surface, thereby stabilising the gas cells (Sahi, 2003).

Fourier transform infrared analysis of a gluten model system consisting of LPC and isolated gluten has provided evidence of the molecular interactions of LPC and gluten at the interface in aqueous conditions (Mohamed et al., 2005). The interaction between LPC and gluten facilitates the ability of gluten to form the network, which is essential for dough formation. As well as interacting with the continuous aqueous component of dough, LPC will interact with gluten via hydrophobic bonds. This may facilitate the physical action whereby gluten extends to cover more dough surface and as a result increases gas retention.

5.5 Interactions of specific lipid-binding proteins with lipids

Purothionin, ligolin, S protein, CM protein, PIN and LTP have been classified as lipid-binding proteins. Isolation methods and chemical characteristics for these proteins have been discussed in Chapter 4. Purothionin, ligolin, S protein and CM protein have been identified as having lipophilic properties. It has been shown that PIN and LTP are able to associate with lipids and to contribute to the foaming properties of an aqueous phase from dough, resulting in the enhancement of both crumb texture and loaf volume. The lipid-binding properties of these proteins will be discussed later.

5.5.1 Purothionin, S protein and CM protein

Purothionin, isolated from a light petroleum extract of flour, has been found to be associated with polar lipids (Redman and Fisher, 1968; Hoseney et al., 1970b). The composition of lipid in lipid-purothionin complexes depends on the isolation method.

After precipitation with methyl acetate solution, followed by separation on a Sephadex LH-20 column with an ethanol-chloroform mixture, purothionin was primarily associated with PC and other polar lipids (Redman and Fisher, 1968). However, a sequential precipitation with butanol, acetone and finally ethyl-acetate results in purothionin forming complexes primarily with mono- and digalactosyl glycerols, PE and PC (Hoseney et al., 1970b).

In order to verify the composition of lipids associated with purothionin, interaction studies have been performed using a model solution containing pure purothionin fraction and different lipid components (Lasztity, 1996). The strongest interaction was found between purothionin and polar phospholipids, probably on side chains of arginine and lysine. Glycolipids may be integrated into the complex only if the protein-phospholipid is formed first. In spite of this, questions remain about the formation of lipoprotein complexes involving purothionin in wheat (Carr et al., 1992), as no technical role has been found for this protein in baking (Hoseney et al., 1970b). However, thionins have been found to involve in altering lipid solubility as well as redox potentials in chemical reactions. Therefore, they likely affect the relationships that exist between lipids and baking performance (Jones et al., 2006)

Another lipid-binding protein, named ligolin, was isolated from the final supernatant in the selective precipitation of AUC-soluble gluten proteins with ammonium sulfate. A significant proportion of labelled lipid remained in the supernatant with a LMW protein (9 kDa) (Frazier et al., 1981; Chung, 1986). This group of proteins, with a large number of cysteine residues, could be responsible for lipid binding during dough development.

S protein, prepared by the fractionation method used by (Frazier et al., 1981), has been found to associate with approximately 20% of the polar lipid in flour (Zawistowska et al., 1985). The characteristics of S protein are similar to CM protein extracted by solutions of chloroform and methanol (Meredith et al., 1960). The evidence of CM protein-lipid interaction is based on the accumulation of protein at the interface of lipid extracts. However, these observations could be due to the preparation process for the protein, rather than the lipid and protein association (Carr et al., 1992).

5.5.2 Puroindolines

PIN, a surface-active protein, exhibits a high affinity with lipids, especially those which are polar, including phospholipids (Kooijman et al., 1997) and glycolipids (Dubreil et al., 1997). They can only interact with a lipid interface and not with monomeric lipids. Both hydrophobic and electrostatic bonds are responsible for the interaction of PIN with polar lipids (Marion et al., 2003). The structure of the tryptophan-rich region in PIN (Chapter 4) plays a major role in the interaction with lipids, influencing the strength of interactions with different types of lipid.

Interactions of PIN and water-soluble model lipids, including phospholipids, were investigated by fluorescence emission and CD spectroscopy (Kooijman et al., 1997). A loop, formed in a tryptophan-rich domain within PIN, forces this region into a conformation that is advantageous for lipid binding. The long alkyl chain of lipid molecules (C16) is sufficient to allow hydrophobic interaction with protein. An electrostatic interaction between the positively charged amino acids in the tryptophan-rich domain and the negative charge of the head group of lipids enhances the strength of binding to PIN. The hydrogen bond between the hydroxyl group of the glycol of the lipid head group and amino acid residues in the lipid-binding domain of the protein may contribute to the binding of lipids and PIN.

Two isoforms of PIN, PIN-a and PIN-b, exhibit different lipid-binding capacities, due to the loss of tryptophan residues of the PIN-b molecular structure (Dubreil et al., 1997). PIN-b displays a higher positive net charge than PIN-a, interacting more strongly with anionic lipids than PIN-a. Due to a difference of net charge on their molecules, PIN-a is capable of binding tightly to both wheat phospholipids and glycolipids, whereas PIN-b interacts strongly only with negatively charged phospholipids, forming loose lipoprotein complexes with glycolipids. Ionic, hydrogen and hydrophobic bonds, which can be formed from the indole ring of tryptophan, contribute to the stability of complexes between PIN and a polar lipid. The truncated tryptophan-rich domain in PIN-b is probably responsible for the low affinity of PIN-b with glycolipids. The highest affinity of PIN-a for glycolipids and neutral LPC could be primarily due to the formation of

hydrogen bonds between glycolipid hydroxyls or carbonyls and indole amine groups (Dubreil et al., 1997).

Furthermore, the interfacial properties of spread monolayers of PIN-a and PIN-b have been studied at varying sub-phase compositions using a Langmuir-Blodgett film balance (Biswas et al., 2001). It was observed that both PIN-a and PIN-b form stable monolayers at the air-water interface. The stability of the monolayers was dependent on the sub-phase composition and the concentration of protein in the spreading solution. When the ionic strength was above 0.5, an increase of ionic strength significantly affected the compression isotherms for both PIN-a and PIN-b. At the same level of ionic strength, $CaCl_2$ had a significant effect on the level of the compression isotherm when compared with NaCl and KCl. A shift of the pH of the sub-phase from 7.2 to 3.0 did not affect the value of collapse pressure but decreased the value of limiting area.

As surface-active proteins, PIN are able to form very stable foams, which are ten times more stable than egg-white protein foams and highly resistant to destabilisation by both neutral and polar lipids. The presence of polar lipids exhibited a synergistic enhancement on the stability of the PIN foams. In beer, a small amount of PIN can restore foam that has been destabilised by various neutral and polar lipids. In bread, PIN can contribute to the formation of crumb with a homogeneous structure composed of fine gas cells. Addition of PIN to wheat flour induced an increase in dough tenacity and a decrease in dough extensibility (Douliez et al., 2000a).

An investigation of interactions between PIN-a and lipid in bread dough using confocal scanning laser microscopy (Dubreil et al., 2002) has demonstrated that lipids are located around gas cells and within the protein-starch matrix, whilst PIN-a was associated with lipid, primarily in the matrix of dough. On the other hand, in defatted dough, PIN-a was found around the gas cells.

5.5.3 Lipid-transfer proteins

LTP are able to facilitate the transfer of lipids between membranes. Some are specific and others are non-specific but all exhibit a binding ability for a broad range of lipids including phospholipids, glycolipids, fatty acids and sterols. There are two types of

nsLPT including nsLPT1 and nsLPT2, the properties and chemical structure of which have been reviewed in Chapter 4. Many studies of lipid-binding properties have concentrated on the nsLTP1, whilst the interactions of LTP2 with lipids have not yet been characterised (Marion et al., 2003).

The binding properties of nsLTP1 have been particularly studied by the structural determination of the lipid-protein complex. It has been found that the volume of a tunnel on the nsLTP1s can adapt for binding one or two monoacylated lipids (Shin et al., 1995; Charvolin et al., 1999) or a diacylated lipid (Sodano et al., 1997). The tunnel has a high plasticity; therefore the binding properties of plant nsLTP1s could not be predicted on only the basis of the volume of the tunnel of the free protein (Douliez et al., 2000a).

The structure of nsLTP1 and lipid complexes could provide information on how the lipid enters the protein. The nsLTP1 can associate with lipid in vesicles or on micelles, hence the first interaction between protein and lipid is polar and electrostatic. It has been suggested that the C-terminal region could play a role in the binding process because few amino acids appear to be mobile in the crystal structure (Shin et al., 1995). Therefore, the mechanism of lipid binding could involve the opening of the C-terminal region exposing a hydrophobic cavity, allowing the lipid to be taken up within the protein molecule (Douliez et al., 2000a).

In associating with polar lipids, the lipid-protein complex is stabilised by hydrogen bonds between the tyrosine of the C-terminal region and the lipid phosphate or carboxylate group and also by the interaction between anionic lipids and the arginine residue (Douliez et al., 2000a). In a complex with lipids, such as oleate and caprate, the lipid-binding ability of nsLTPs is due to the presence of a hydrophobic cavity in their molecules (Han et al., 2001). There are probably two sites on wheat nsLTP which allow lipids to insert head to tail from opposite ends of the hydrophobic cavity. ns-LTP is able to interact with both monoacyl and diacyl lipids by forming micelles or lamellar liquid-crystalline phases (Han et al., 2001).

The interaction of 1,2 dimyristoylphosphatididyl glycerol (DMPG) and ns-LTP occurs at the bilayer interfaces as well as inside the internal cavity of the protein (Sodano et al.,

1997). The volume of the hydrophobic cavity of maize nsLTP can vary depending on the size of the ligand, therefore, introducing a bulky lipid such as DMPG into ns-LTP does not significantly deform the protein structure. Although three residues (Tyr81, Arg46 and Asn37) are responsible for interaction with the carboxylate group of the lipid molecule, the hydrogen bond between the hydroxyl of Tyr81 and the carbonyl of the lipid was not found in the DMPG complex (Sodano et al., 1997).

For nsLTP1, two lyso-myristoylphosphatidylcholine (LMPC) molecules are able to insert head-to-tail in the hydrophobic cavity. This protein contains two lipid-binding sites having differing affinities for lipids. At site 1, LMPC strongly interacts with the protein through hydrophobic interactions and a hydrogen bond, while at site 2, LMPC is involved only in hydrophobic interactions (Charvolin et al., 1999).

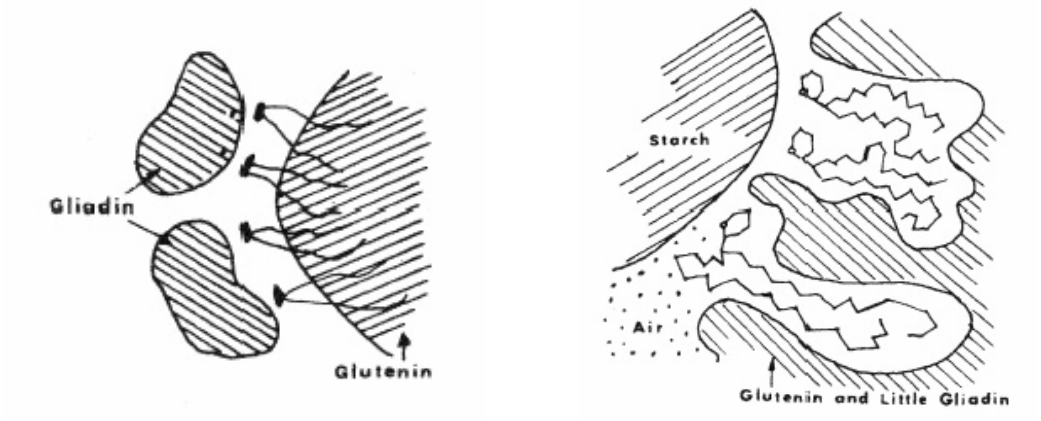
The affinity of protein and lipid are characterised by the dissociation constant that can be measured by following the increase of intrinsic tyrosine fluorescence during lipid binding. The capability of nsLTP1 to bind different mono- and di-acylated lipids was confirmed with a tyrosine fluorescence titration (Douliez et al., 2000b). Measurements of the number of binding sites of these proteins range between 1.6 and 1.9, suggesting that two lipids can fit within the protein. With this technique, it was shown that barley LTP1 was capable of binding two mono-acylated lipids or two ω-hydroxypalmitic acid molecules on its small hydrophobic cavity (Douliez et al., 2001b).

5.6 Reviewing the proposed mechanisms of protein-lipid interactions

Various proposed mechanisms of protein-lipid interactions in breadmaking have been reported previously (Chung, 1986; Carr et al., 1992), primarily based on the results of a number of studies using chemical and physical techniques. There is considerable evidence for the occurrence of chemical interactions between protein and lipid in dough and gluten (Hoseney et al., 1970a; Wehrli and Pomeranz, 1970; Chung, 1986) while several studies provided evidence of the existence of lipid in the liposomes or vesicles in dough and gluten structure (Larsson, 1985; Marion et al., 1987). The mechanisms of protein and lipid interaction in dough and gluten have not been fully resolved and these depend on the approaches used in the study.

From the distribution of lipid in the glutenin and gliadin fractions, (Hoseney et al., 1970a) proposed the existence of a gliadin-glycolipid-glutenin complex (Figure 5.1, A), in which glycolipids are associated with gliadin through hydrophilic links and with glutenin through hydrophobic bonds. Investigating complexes between glycolipids and raw starch, gelatinised starch, gliadin and glutenin, (Wehrli and Pomeranz, 1970) indicated the presence of hydrogen bonds between glycolipids and gelatinised starch or gluten components, and van der Waals bonds between glycolipids and gluten components. On this basis, they proposed a model for a starch-glycolipid-gluten complex in the dough system (Figure 5.1, B).

Lipid has been observed to occur as a lamellar, liquid crystalline phase in dough, and so it has been suggested that it is organised into the phase structure and does not become involved in molecular interactions with protein (Larsson, 1985; Marion et al., 1987; Carr et al., 1992). Lipid could be physically entrapped into the gluten matrix and interact with protein by polar and ionic bonding on the surface of the lipid phase (Carr et al., 1992).



(A) (B)

Figure 5.1 Proposed models of protein and lipid interaction in dough

Notes (A) Gliadin-glycolipid-glutenin complex (Hoseney et al., 1970a)
(B) Starch-glycolipid-gluten complex (Wehrli and Pomeranz, 1970)

A model of foam stability has been proposed to provide an understanding of the role of lipid in baking (MacRitchie, 1983). Initially, this model was not intended to investigate the interaction of protein and lipid. Subsequently, microstructural studies have shown evidence regarding the interaction of polar lipids with gluten proteins on the gas cells,

and these may contribute to the stabilisation of foams during baking (Keller et al., 1997; Paternotte et al., 1994; Li et al., 2004).

Lately, the interactions of lipid-binding proteins (PIN and LTP) and lipids have been of interest. Several models of protein-lipid interaction have been proposed, showing that PIN and LTP interact with lipid on a molecular level; however, PIN can only associate with a lipid interface, while LTP interact with lipid in vesicles or micelles (Marion et al., 2003). PIN can interact with polar lipid through ionic, hydrogen and hydrophobic bonds, whilst lipid molecules can insert into a tunnel in the LTP structure and structural stability is provided by the hydrogen bonds and hydrophobic interactions (Section 5.5).

More recently, evidence for the molecular interactions of phospholipid and gluten has been observed through the LPC and an isolated gluten model (Mohamed et al., 2005). Phospholipid can interact with gluten by hydrophobic interaction, therefore assisting gas retention.

5.7 Summary of the current literature on protein-lipid interactions

The occurrence of protein and lipid interactions in dough and gluten, and their important role in contributing to the quality of bakery products, has been recognised. However, knowledge on the mechanism of these interactions remains incomplete. The distribution of lipid in protein fractions indicates that specific lipid groups associate with particular protein fractions, but these associations depend on the fractionation method. In contrast, other studies suggest that there is not a specific interaction between gluten protein and lipid; the molecular protein-lipid interactions are not found, and lipid remains within the gluten network through physical entrapment. However, the study of foam stability and gas cell structure has demonstrated the interaction of polar lipid and protein on the surface of gas cells. Moreover, models of lipid-binding proteins (PIN and LTP) with lipid, and recently the model of isolated gluten and LPC, have indicated the existence of protein and lipid interactions within gluten at the molecular level.

Dough and gluten are complex systems containing various types of proteins and lipids having different properties, and this has resulted in studies of protein-lipid interactions being hampered considerably. These interactions play an important role in baked

products; therefore studies which might further our understanding of these mechanisms are essential for improving the quality of bakery products and extending applications of flour and gluten, as well as the optimal utilisation of the unique functionality of gluten as a bakery ingredient.

Chapter 6

Summary of background and description of project aims

The purpose of this chapter is to summarise the context in which this study has been developed and to describe the aims of the project.

6.1 Summary of current situation and significance of the project

The production of vital wheat gluten globally has increased during the last ten years, currently providing approximately 500,000 tonnes annually. This probably reflects the expansion of gluten applications in both food and non-food products. Gluten is commonly used as an ingredient for bakery products, breakfast cereals and pet foods. Apart from these, gluten finds application as a binding agent for processed meat, a meat substitute for vegetarians and recently a replacement for gelatine in chewy candy. The wide range of applications is due to the fact that gluten has unique functional properties and is relatively inexpensive compared to other protein products, particularly those from milk and soy.

The unique functional properties of gluten are contributed by the two main components of gluten proteins, known as glutenin and gliadin. Glutenin, capable of forming an extended protein network upon gluten rehydration, brings the high viscosity and elastic behaviour to gluten; whilst gliadins, as monomeric proteins, provide the flow properties. It has been suggested that lipid participates in this structure on the basis of the protein-lipid associations. For this reason, it is difficult to remove lipid from gluten without disrupting its structure. The effect of lipid-protein interactions has been studied for many decades, primarily focussing on the physical properties of dough. The moderate amount of lipid in gluten might have an effect on the functional properties of other products having gluten as an ingredient. However, the information on protein-lipid interactions in the gluten complex is limited and further studies are required. Knowledge of this area could provide substantial economic benefit by developing strategies to manipulate the protein-lipid complex and its functional applications as well as enhancing both the sensory attributes and storage characteristics.

6.2 Hypothesis

This project is based upon the hypothesis that the interactions between lipid and protein in gluten might be attributed to the physical entrapment of lipid within gluten structure as well as the association of protein and lipid through non-covalent bonds. Acetic acid is able to affect hydrogen bonds and net charge of protein molecules, resulting in changes to the structure of the gluten matrix. The use of acetic acid would be expected to increase the solubility of at least some proteins, thereby releasing some lipids if these were entrapped within the gluten matrix.

6.3 Project aims

The broad aim of this project has been to investigate the interactions between lipid and protein components during gluten preparation. Based on the hypothesis, the specific objectives have been to:

1) Develop a lipid extraction procedure for gluten and identify lipid classes using HPLC

This phase is essential for establishing the lipid extraction procedure and lipid analysis method that will be used for all other phases of this study. The lipid extraction procedures will be evaluated for their effectiveness in extracting separately free and bound lipid. In addition, the method needs to be suitable for extracting lipid gluten without changing the levels of the lipid components and classes. The validated HPLC method can then be applied to determine the amount of lipid classes in free and bound lipid extracts.

2) Study the effect of acetic acid on lipid and protein distribution

This phase of study aims to investigate the effect of acetic acid on lipid and protein distribution using three different approaches. Firstly, treatment of gluten with various acetic acid concentrations is used to observe the changes in the protein composition of gluten as well as the free and bound lipid distribution in gluten. These changes might provide evidence regarding the physical entrapment of lipid within the gluten matrix.

Secondly, acetic acid at various concentrations will be used to fractionate gluten protein into supernatants and pellets. This fractionation is applied for studying the effect of acetic acid concentrations on the protein composition of the gluten fractions. Accordingly, the distribution of free and bound lipid in each fraction could relate to its protein composition, therefore, indicating the association of particular lipids and proteins in the gluten fractions.

Finally, gluten will be sequentially fractionated in two steps, firstly with a low acetic acid concentration and followed with a higher acetic acid concentration. This fractionation is aimed to obtain gluten fractions with different protein composition. The free and bound lipid distribution in these fractions may provide further evidence of the interactions of protein and lipid in gluten.

3) Identify the presence of specific protein-lipid interactions in gluten

The protein profile of the sequential acetic acid fractions will be further characterised using a variety of methods. These aim to provide additional differentiation of protein composition within these fractions, which can reveal the occurrence of specific protein-lipid interactions. In addition, the treatment of the acetic acid insoluble fraction with reducing agent will be employed to observe the effect of disulfide bonds within the gluten structure on the interactions between protein and lipid.

Chapter 7

Materials and methods

The purpose of this chapter is to describe the chemicals, reagents, equipment and methods used during this study. Included are procedures for gluten preparation, gluten fractionation, lipid extraction as well as methods applied for lipid and protein characterisation. The approaches used in data handling are also highlighted.

7.1 Materials

The chemicals, including lipid standards, protein standards and those used in acetic acid fractionation and analytical procedures were of analytical grade or of the highest purity available, unless otherwise specified. The details of the lipid standards, the protein standards used for electrophoresis and other chemicals used in this study are presented in Tables 7.1, 7.2 and 7.3, respectively. The flour samples investigated were a commercial flour (S008) from year 2002 harvest, provided by Manildra Group (Nowra, NSW) and single variety flours milled from hard wheats (Lang and Sunco cultivars) and a soft wheat (Rosella cultivar), all from year 2004 harvest, provided by Allied Mills (Kensington, VIC). Throughout this study tap water was used only for gluten preparation, while deionised water was utilised for gluten fractionation, protein extraction and general analytical purposes. Milli Q water was used for the analytical procedures involving HPLC and electrophoresis.

Table 7.1 List of lipid standards

Supplier	Lipid standard	Cat no, batch no
Sigma, NSW, Australia	Glyceryl trilinoleate	T9517, 093K5216
	1,3-dilinoleoyl-rac-glycerol	D9505, 113K1341
	1-linoleoyl-rac-glycerol	M7640, 063K1300
	Galactosyl diglyceride (wheat flour)	G9523, 113K4030
	Digalactosyl diglyceride (wheat flour)	D4651, 103K4052
	L-phosphatidylcholine (soybean)	L0906, 064K5216
	Lysophosphatidylcholine (soybean)	P7443, 76H8377
	L-phophatidylethanolamine (soybean)	P8193, 103K5214
	Lysophosphatidylethanolamine (egg yolk)	L4754, 122K0374
	L-phophatidyl L-serine (soybean)	P0474, 074K5210
	L-phosphatidylinositol ammonium salt (soybean)	P5954, 044K5204

Table 7.2 List of protein and lipoprotein standards

Supplier	Standards	Cat no, batch no
Invitrogen, Melbourne, Australia	Mark 12 unstained standard contains: - myosin (MW: 200 kDa), - β-galactosidase (MW: 116.3 kDa), - phosphorylase B (MW: 97.4 kDa), - bovine serum albumin (MW: 66.3 kDa), - glutamic dehydrogenase (MW: 55.4 kDa), - lactate dehydrogenase (MW: 36.5 kDa), - carbonic anhydrase (MW: 31.0 kDa), - trypsin inhibitor (MW: 21.5 kDa), - lysozyme (MW: 14.4 kDa), - aprotinin (MW: 6.0 kDa), - insulin B chain (MW: 3.5 kDa), and - insulin A chain (MW: 2.5 kDa)	LC5677, MRK50726
Invitrogen, Melbourne, Australia	NativeMark unstained protein standard contains: - IgM hexamer (MW: 1236 kDa), - IgM pentamer (MW: 1048 kDa), - Apoferritin band 1 (MW: 720 kDa), - Apoferritin band 2 (MW: 480 kDa), - B-phycoerythrin (MW: 242kDa), - Lactate dehydrogenase (MW: 146 kDa), - Bovine serum albumin (MW: 66 kDa), and - Soybean trypsin inhibitor (MW: 20 kDa)	LC0725, 1321528
Sigma, NSW, Australia	Albuminbovin fraction V (bovine serum albumin)	A-3294, 68H1101
Helena Laboratories, Beaumont, Texas	LipoTrol (mixture of lipoprotein standards)	000469, 2-04-5069

Table 7.3 Detail of chemicals and suppliers

Supplier	Chemicals
Ajax Fine Chemicals, NSW, Australia	Acetic acid glacial Univar (UN No 2789, AH 503218); Orthophosphoric acid 85% (371, 108205); Potassium chloride KCl (A383, AF602436); Sudan black (C8690, A15B);
B&J, USA	Dichloromethane (HPLC grade, AH300-4, 10071756)
BDH, Australia	Ethylene diaminetetra acetic acid disodium salt – EDTA (10093.5V, 28331); Chloroform (10077.6B, 34962); Petroleum spirit or petroleum ether (10178.4F, 35321)
BDH, England	Hydrochloric acid (36%) (101256J, K32772152-350); Methanol Hipersol for HPLC (15250, L458602); Sodium dihydrogen orthorphosphate dihydrate $NaH_2PO_4.2H_2O$ (301324Q, K91551420142); Sodium dodecyl sulfate – SDS (301754L, ZA9543710909); Sulfuric acid (~90%) (303246E, Z446207-737); Trifluoroacetic acid – TFA (15311.2E, 113152)
Bio-Rad Laboratories Ltd, NSW, Australia	Acrylamide solution 40% (161-0140, 200001062); Coomassie Blue G-250 (161-0406, 162147A); DC protein assay-reagent A (500-0113, 210001776); DC protein assay-reagent B (500-0114, 210001774)
Crown Scientific Ltd, Victoria, Australia	Diethyl ether (1.00921.2500, 37879); Disodium hydrogen orthophosphate dihydrate $Na_2HPO_4.2H_2O$ (10383, 15412); Isopropanol (LiChrosolv, 1.01040.2500, I222440514); Sodium chloride (SA046, (10)230838); Sodium hydroxide (SA178, (10)230961);
CSR Ltd, Victoria, Australia	Absolute ethanol (UN 1170, Code 100, 7/04); Ethanol 95% (UN 1170, Code 95, 121104)
Invitrogen, Victoria, Australia	Native PAGE 5% G 250 sample additive (BN2004, 1317476); NativePAGE Novex 3-12% Bis-Tris gel (BN2011BX10, 6011880); NuPAGE 4-12% Bis-Tris Zoom Gel 1.0 mm × IPG well (NP0330BOX, 5051760); NuPAGE Antioxidant (NP0005, 1323219); NuPAGE gradient gel (4-12%) Bis-Tris 1.0 mm × 10 well (NP0321BOX, 6090172); NuPAGE gradient gel (4-12%) Bis-Tris 1.0 mm × 17 well (NP0309BOX, 6032370); UltraPure Agarose (15510-019, 1259532); UltraPure Dithiothreitol (15508-013, 26722); Zoom Carrier Ampholytes pH 3-10 (ZM0021, 01261584); Zoom CHAPS (ZC10003, 389017-0705); Zoom strip pH 3-10 NL (ZM0011, 1254958); Zoom Urea (ZU10003, 388702-0705);

Table 7.3 Detail of chemicals and suppliers continued

Supplier	Chemicals
J.T. Baker, USA	Acetonitrile (HPLC grade, 9017-03, 013110532G24)
John D. Bolton, UK	Coomassie Brilliant Blue R-250 (3195, 81281B)
Selby Biolab, Melbourne, Australia	Glycerol (GL 885, 23085)
Sigma, NSW, Australia	2,2,4-Trimethylpentane (HPLC grade, 27,035-0, BC15243BC); Bis-Tris (B9754, 095K5400); Bromophenol Blue (B0126, 94C-0313); Guanidine hydrochloride (G4630, 045K0190); Hydroxypropyl methyl cellulose – HPMC (H7509, 045K0051); MES [2-(N-morpholino) ethane sulfonic acid] monohydrate (69892, 444347/1); N-ethylmorpholine 99% (E0252, 39H1087); Thiourea (T7875, 76H0905); Tricine (T0377, 121F-5040); Tris (hydroxymethyl) aminomethane 99.8+% ACS reagent (252859, 01401EB); Triton X114 (X114, 035K0203);

Note Description presented as chemical name (product number, batch or lot number)

7.2 Apparatus and auxiliary equipment

The items of equipment used, together with the details of manufacturers and model numbers are presented in Table 7.4. The HPLC instrumentation applied for protein analysis and lipid analysis are described in Tables 7.5 and 7.7, respectively. The columns and ancillary items used for protein analysis and lipid analysis on HPLC are detailed in Tables 7.6 and 7.8.

Table 7.4 Description of equipment and instrumentation

Equipment	Manufacturer / supplier	Model no
Assay microplate 96 wells, Flat bottom	Iwaki, Japan	Code 3881-096 Lot no 1990504
Balance	Mettler, Switzerland	AE 200-S Serial L35304
CAP capillary tubing	Beckman Coulter, USA	Uncoated silica capillary (50 μm ID, 37.5 μm OD) Order no 338472
Capillary Electrophoresis Cartridge	Beckman Coulter, USA	P/ACE System MDQ Order no 144738
Capillary Electrophoresis System	Beckman Coulter, USA	P/ACE MDQ Serial no 3061905
Dynavac freeze-drier	Airvac Engineering Pty Ltd	Model FD-5
Electrophoresis cell	Invitrogen, Victoria, Australia	Xcell Novex Serial no 920554
Eppendorf centrifuge	Sigma Ltd, Germany	1-15 Year 2001 Serial no 80472
Evaporator	Genevac Ltd, England	HT-4 series II system Serial no 5608
Gel image analysis software	Bio-Rad, NSW, Australia	Quantity One-4 Version 6.1
Grinder	Breville, China	CG-2
Hobart mixer	Hobart Corporation, USA	N-50G
Leco-FP2000	Leco Corporation, USA	602-600-400 Serial no 3494
Microplate spectrophotometer	Molecular Devices, USA	Spectra Max Plus 384 Serial no MN03510
Multi Reax Shaker	John Morris Scientific, Victoria, Australia	Heidolph
Orbital Shaker	Chiltern Scientific	SS70 Serial no 5835
Power Supply	Bio-Rad Laboratories, NSW, Australia	160/1.6 Serial no 54BR1175
Prot/Elect tips Bulk 1000 tips	Bio-Rad Laboratoies Ltd, NSW, Australia	Cat no 223-9915

Table 7.4 Description of equipment and instrumentation continued

Equipment	Manufacturer / supplier	Model no
Reference pH meter	Radiometer Pacific, Australia	PHM93
Rotary evaporator	Büchi Labortechnik, Germany	Büchi Rotavapor R-124
Ultra-Turrax	Janke & Kunkle IKA-Labortechnik, Germany	T25 Serial no 3494
Vacuum drier	Genevac Ltd, Ipswich, England	Model HT-4 Series II
Vortex mixer	Ratek Instrument, Victoria, Australia	Model VM1 Serial no 50393274
Water bath	Ratek Instrument, Victoria, Australia	WB20 Serial no 2119695
ZOOM IPG Runner	Invitrogen, Victoria, Australia	Max Voltage 3500 V, Max Wattage 3.5 W Serial no 0410235
ZOOM IPG Runner Cassette	Invitrogen, Victoria, Australia	Cat no ZM0003 Lot no 1251064

Table 7.5 Description of HPLC system components for protein analysis

Component	Manufacturer / supplier	Model no
Quaternary pump	Shimadzu, Japan	LC-10Ai Serial no L20304200313SL
Control Panel	Shimadzu, Japan	CBM-20A Serial no L2023420119CD
Autosampler	Shimadzu, USA	SIL-20AC Serial no L20174370008USD
UV/VIS detector	Shimadzu, Japan	SPD-20A Serial no L20134301221LP
Fraction collector	Shimadzu, Japan	FRC-10A Serial no C20374304150SA
Data handling system	Shimadzu, Japan	Class VP Version 7.3

Table 7.6 Description of columns and ancillary items used in HPLC analysis of proteins

Item	Manufacturer / supplier	Model no
BioSep SEC S4000 300 × 7.80 mm	Phenomenex, NSW, Australia	Part no OOH-2147-KO S/No 195654
BioSep SEC S4000 Guard column 35 × 7.8 mm	Phenomenex, NSW, Australia	Part no 030-2147-KO S/No 195655G
Jupiter C18, 5 μm, 250 × 4.60 mm	Phenomenex, NSW, Australia	Part no OOG-4053-EO S/No 332235-8
Security Guard Guard Cartridge	Phenomenex, NSW, Australia	Part no KJO-4282
Widepore C18 (ODS) 4.0 mm L × 3.0 mm ID	Phenomenex, NSW, Australia	Order no AJO-4321
Syringe filters PES 0.45 μm, 30 mm	Bonnet, Melbourne, Australia	Order no 30PS045AN
Syringe filters Nylon 0.45 μm, 30 mm	Bonnet, Melbourne, Australia	Order no 30NP045AN
Membrane filter-Magna, Nylon, supported, plain, 0.45 μm, 47 mm	Bonnet, Melbourne, Australia	Order no R04SP047BN
Membrane filter, mixed cellulose ester, 0.45 μm, 47 mm	Bonnet, Melbourne, Australia	Order no A045A047A

Table 7.7 Description of HPLC system components used for lipid analysis

Component	Manufacturer / supplier	Model no
Quaternary pump	Hewlett Packard, Japan	HP 1050
Degasser	Hewlett Packard, Japan	G1303A Serial no 3227J01303
Autosampler	Hewlett Packard, Germany	G1306AX Serial no 3343G00302
PL-ELS detector	Polymer Laboratories, UK	PL-ELS 1000 Serial no 003-1022
Data handling system:	Agilent Technology, Australia	Chem Station for LC 3D Rev A.09.03

Table 7.8 Description of columns and ancillary items used in HPLC analysis of lipids

Item	Manufacturer / supplier	Model no
YMC Pack, PVA-Sil NP S-5 μm, 12 nm, 250 × 4.6 mm ID	Shaphire Bioscience, NSW, Australia	Part no A-903-5 NP
YMC PVA-Sil Threaded Guard Cartridge, 120 A, S-5, 4.0 × 23)	Shaphire Bioscience, NSW, Australia	Part no 950-10145
4 mm syringe filter, PTFE, 0.45 μm	Alltech, NSW, Australia	Part no 2395
Membrane filter-Magna, Nylon, supported, plain, 0.45 μm, 47 mm	Bonnet, Melbourne, Australia	Order no R04SP047BN

7.3 Material preparation

7.3.1 Gluten preparation

Gluten was prepared on a lab scale from three flour samples provided by Allied Mills (Kensington), which were milled from single varieties of flour hard wheat (Lang and Sunco varieties) and soft wheat (Rosella variety) from year 2004 harvest. Flour (300 g) was mixed with cool water (180mL) for 1 min at setting 1 using a Hobart mixer, and then at setting 2 for 2.5 min to form a dough. This was placed in cool water for 30 min to allow the gluten matrix to further develop and then hand washed with 3 × 5 L of tap water to remove starch. The wet gluten was collected, freeze-dried and then ground to a powder using the mechanical grinder. The dried gluten was stored at –20°C prior to further analysis.

7.3.2 Petroleum ether defatted gluten

Gluten (50 g) was mixed with petroleum ether (200 mL) for 15 min on a magnetic stirrer at moderate speed (MacRitchie, 1985). The slurry was filtered with a Buchner funnel using a Whatman No 45 paper to collect the powder. This process was repeated a further 3 times in order to remove the free lipid. The resultant powder was placed in a

fume cupboard for 3 days to allow evaporation of solvent residue. The defatted gluten was stored at –20°C and used either for further treatment with acetic acid or subjected to fractionation procedures.

7.3.3 Petroleum ether defatted gluten treated with acetic acid

Solution preparation

Acetic acid 1 M (stock solution): Glacial acetic acid (99.9%, 28.57 mL) was mixed with water (approximately 200 mL) and then diluted to 500 mL.

Acetic acid 0.01 M: Acetic acid (1 M, 10 mL) was diluted to 1,000 mL.

Acetic acid 0.05 M: Acetic acid (1 M, 50 mL) was diluted to 1,000 mL.
Acetic acid 0.1 M: Acetic acid (1 M, 100 mL) was diluted to 1,000 mL.

These solutions were also used for the single acetic acid fractionation (Section 7.2.4) and the sequential acetic acid fractionation (Section 7.2.5)

Procedure for acetic acid treatment of gluten

Each defatted gluten sample (10 g) was mixed with 0.01 and 0.1 M acetic acid (200 mL) for 2 min using an Ultra Turrax at speed of 9,500 rpm, then freeze dried, ground to powder and stored at –20°C. The yield of treatment was calculated using the following formula:

$$\text{Treatment yield (\%)} = \frac{\text{Weight of acetic acid treated gluten}}{\text{Weight of gluten control (g)}} \times 100$$

7.3.4 Single acetic acid fractionation of gluten

The procedure for the single acetic acid fractionation of gluten is described in Figure 7.1. Each gluten control sample (10 g) was separately mixed with 0.01, 0.05 and 0.1 M

acetic acid (200 mL) for 2 min using an Ultra Turrax at a speed of 9,500 rpm followed by centrifugation at 24,000 × g, at 4°C for 15 min (Berot et al., 1994). The supernatant and pellet were collected, freeze dried, ground and stored at –20°C. The yield of each acetic acid gluten fraction was calculated using the formula:

$$\text{Yield (\%)} = \frac{\text{Weight of acetic acid gluten fraction}}{\text{Weight of gluten control (g)}} \times 100$$

7.3.5 Sequential acetic acid fractionation of gluten

The procedure for sequential acetic fractionation of gluten is presented in Figure 7.2. Gluten control was fractionated firstly with 0.01 M acetic acid (Berot et al., 1994) at a ratio 1:20 for 2 min using an Ultra Turrax at 9,500 rpm. Supernatant was separated by centrifugation (24,000 × g, 4°C, 15 min). The insoluble substance was then fractionated with 0.1 M acetic acid using the same conditions as those of the first fractionation. Pellet and supernatants of the first and second fractionations were separately freeze dried, ground and stored at –20°C. The yield of each fraction was calculated using the following formula:

$$\text{Yield (\%)} = \frac{\text{Weight of acetic acid gluten fraction}}{\text{Weight of gluten control (g)}} \times 100$$

7.3.6 Dithiothreitol treatment of the insoluble acetic acid fraction

Acetic acid insoluble fraction (the pellet from the sequential acetic acid fraction, 5 g) was mixed with 50 mM dithiothreitol (100 mL) for 1 hr and then vacuum dried using the Genevac evaporator set at 30°C, full vacuum, for 20 hrs. Dried sample was ground and stored at –20°C prior to further analysis.

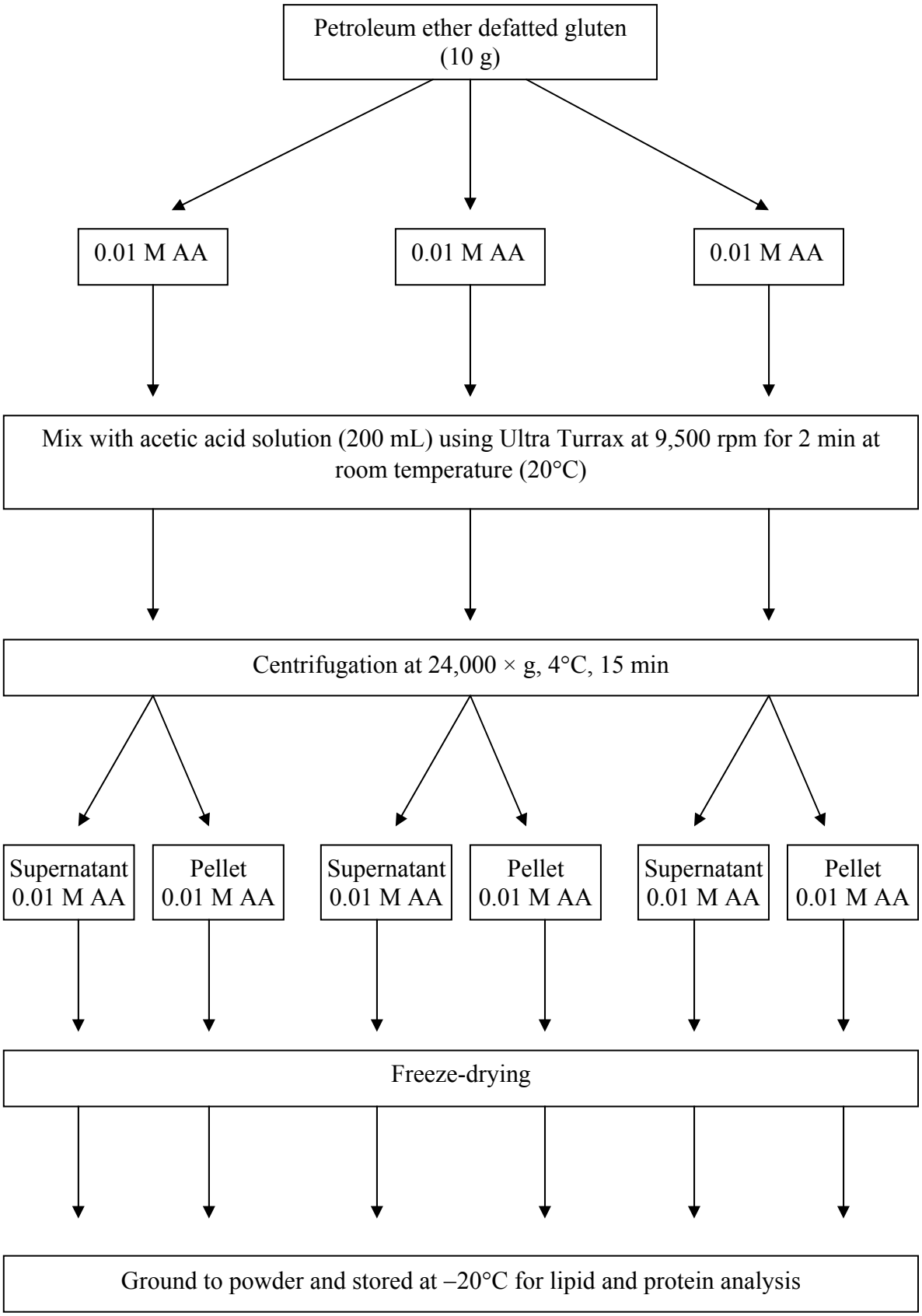


Figure 7.1 Procedure for single acetic acid fractionation
Notes: AA represents acetic acid

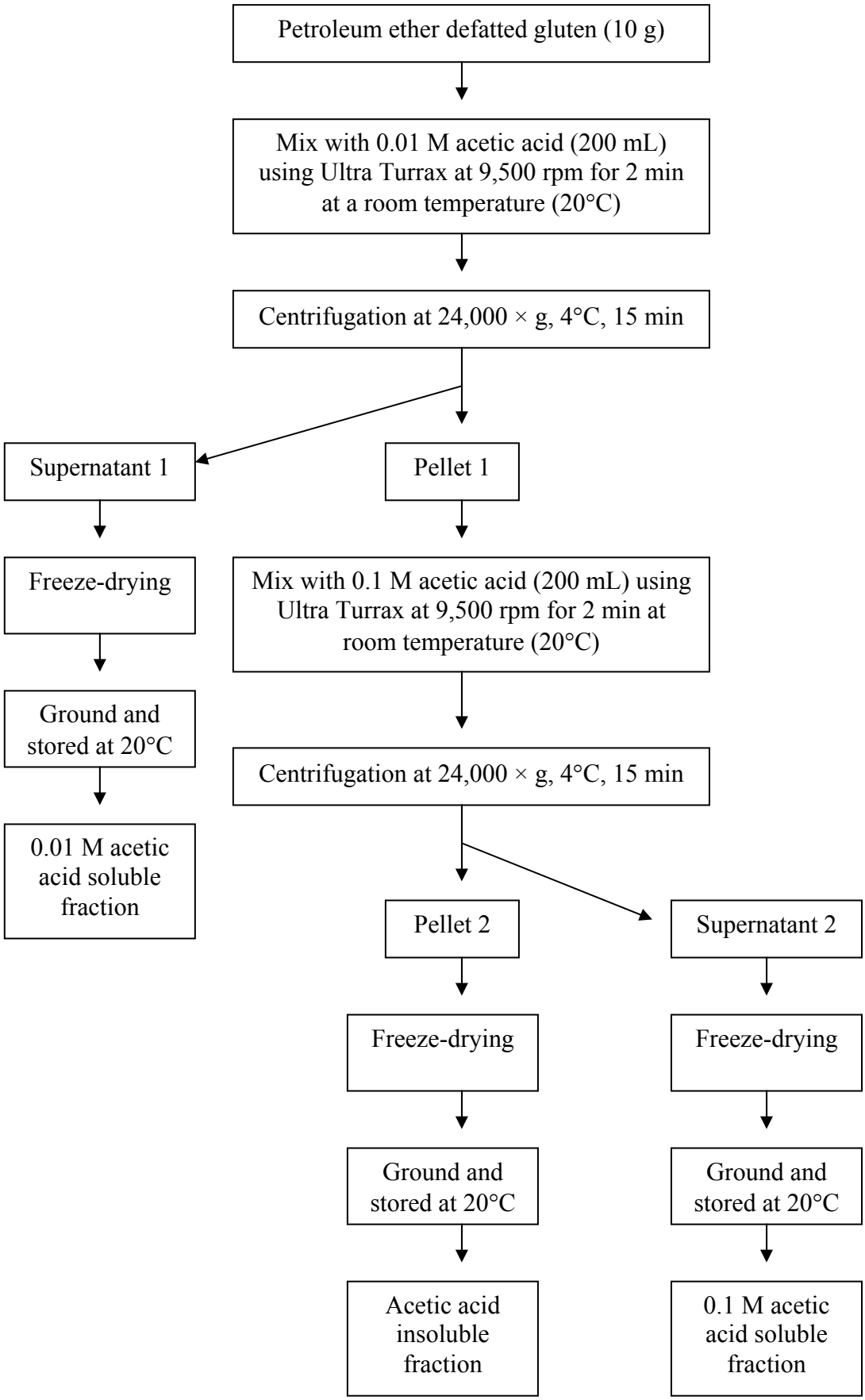


Figure 7.2 The procedure used for sequential acetic acid fractionation

7.3.7 TX114 protein partition of the sequential acetic acid fractions

Solution preparation

Tris-HCl 100 mM buffer, pH 7.8: Tris base (12.114 g) was dissolved in 800 mL of deionised water, adjusted pH to 7.8 using hydrochloric acid (32%) and then diluted to 1,000 mL.

Tris-KCl 100 mM buffer (Tris-HCl 100 mM buffer, pH 7.8 containing 5 mM EDTA and 0.1 M KCl): EDTA disodium salt (1.861 g) and KCl (7.455 g) were dissolved to 1,000 mL using Tris-HCl 100 mM buffer, pH 7.8.

1% TX114 (w/v) in Tris-KCl 100 mM buffer: TX114 (10 g) was dissolved in 1,000 mL of Tris-KCl 100 mM buffer.

4% TX114 (w/v) in Tris-KCl 100 mM buffer: TX114 (40 g) was dissolved in 1,000 mL of Tris-KCl 100 mM buffer.

Diethyl ether-ethanol (1:3, v/v): Diethyl ether (100 mL) was added to ethanol (300 mL) and mixed well.

Procedure of gluten protein extraction using TX114

TX114 protein extraction was performed using the method described by Blochet et al. (1993) and Day et al. (1999). The extraction included steps of mixing and centrifugation and is illustrated in Figure 7.3. Gluten sample or fraction (600 mg) was gently mixed with 20 mL of 4% TX114 (w/v) in Tris-KCl 100 mM buffer at 4°C for 1 hr using a magnetic stirrer and then centrifuged at 2,000 × g for 10 min. The pellet was collected, washed 3 times with deionised water and then freeze-dried. The supernatant was placed in a water bath (30°C) for 1hr and then centrifuged at 2,000 × g for 10 min. Solution was separated into two phases: the upper phase was removed by pipette and discarded. The lower phase was retained and 20 mL of 1% TX114 (w/v) in Tris-KCl 100 mM buffer added, followed by gentle mixing at 4°C for 30 min. The second phase partition was performed by warming the solution in a 30°C water bath for 1hr, followed by

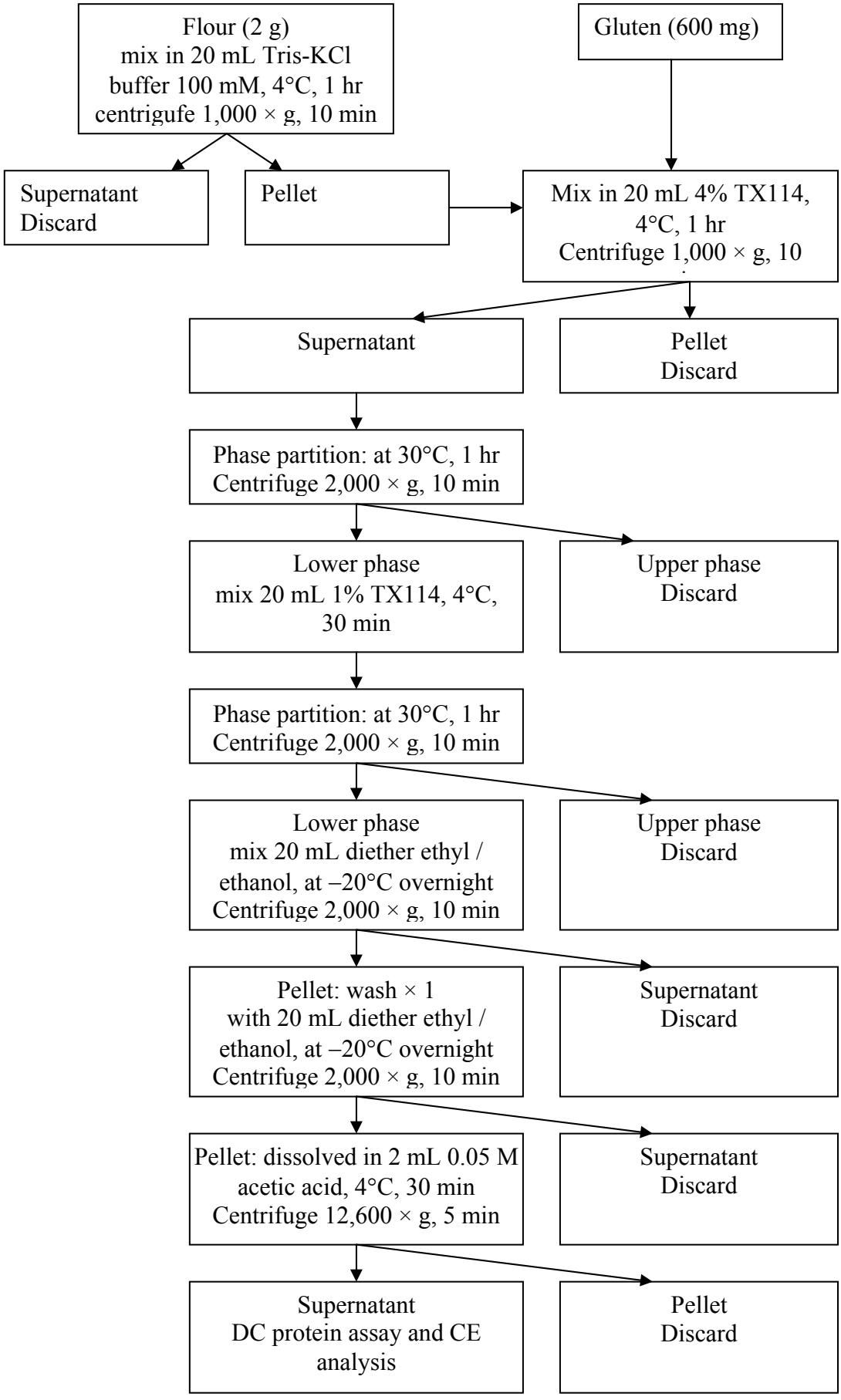


Figure 7.3 The procedure for extraction of protein using TX114

centrifugation. After the upper phase was discarded, diethyl ether-ethanol (1:3, v/v, 20 mL) was added to the lower phase, mixed well and placed in the −20°C freezer overnight to facilitate protein precipitation. Following centrifugation (2,000 × g, 10 min) the pellet was collected and washed with another 20 mL of diethyl ether-ethanol (1:3, v/v, −20°C). The pellet was placed in a fume-hood until the solvent was completely removed. In order to dissolve the soluble protein, the pellet was mixed with 5 mL of 0.05 M acetic acid at 4°C for 30 min and centrifuged at 12,600 × g for 5 min. The supernatant was collected for protein analysis using capillary electrophoresis (described in Section 7.6.6) and DC protein assay (Section 7.6.7).

For flour, a 2 g sample was used in the extraction and an additional step was required to remove water-soluble proteins before carrying out the TX114 extraction. This step involved mixing flour with 20 mL of Tris-KCl 100 mM buffer at 4°C for 1 hr and centrifugation at 1,000 × g for 10 min. The pellet was retained and used for the TX114 extraction.

7.4 General procedures for testing of flour and gluten

7.4.1 Moisture content

Moisture content of flour and gluten sample was determined using AACC method 44-15A (AACC, 1995b). A sample of Flour or gluten sample (1–2 g) was weighed into an aluminium moisture dish (7.2 cm diameter), covered with the lid at once and the weight of sample recorded. The weight of the moisture dishes containing sample and lid were also recorded. The dishes were uncovered and placed in an oven at 103°C together with the lids for 60 min after oven recovered the temperature. The lids were placed onto the respective dish and transferred to a desiccator as quickly as possible. When the dishes reached room temperature (approximately 60 min), the weight of dishes was recorded and the moisture content of sample was calculated using the equation presented below. The drying process was continued until the readings were within 0.2% moisture. The resultant values were used in order to express other values on a dry matter basis in order to facilitate direct comparisons.

$$\text{Moisture content (\%)} = \frac{\text{A (g)}-\text{B (g)}}{\text{Amount of sample (g)}} \times 100$$

Note: A is the weight of dish and sample before heating in an oven
B is the weight of dish and sample after heating in an oven

7.4.2 Total protein analysis

The protein contents of gluten and gluten fraction samples were analysed with a Leco FP-2000 Nitrogen/Protein analyser using the AACC method 46-30 (AACC, 1995c).

Procedure of total protein analysis on Leco FP-2000 and calculation

Calibration: This was conducted each day prior to analysis of samples using EDTA as a calibration standard. The amount of EDTA ranged between 0–180 mg, which is equivalent to amounts of nitrogen from 0–17 mg.

Sample measurement: Flour, gluten or gluten fraction (0.080–1.000 g) was weighed into a ceramic boat and stored in a desiccator until the instrument was available. The amount of sample was selected to correspond with an amount of nitrogen within the range of the calibration. Sample boats were stacked in the rack and placed into the sample chamber. The temperature set for the combustion process was 1,100°C followed by cooling to 200°C.

Calculation:
The protein content in flour, gluten or gluten fraction samples were calculated as follows:

$$\text{Protein content (\% or g/100 g of sample)} = \frac{\text{Amount of nitrogen (mg)} \times 5.70}{\text{Amount of sample (g)} \times 1{,}000} \times 100$$

The protein recovery from individual acetic acid gluten fractions was calculated using this formula:

$$\text{Protein recovery (\%)} = \frac{\text{Protein content of fraction (\%)} \times \text{weight of fraction (g)}}{\text{Protein content of gluten (\%)} \times \text{weight of gluten (g)}} \times 100$$

7.5 General procedure for lipid analysis in flour, gluten and gluten fractions

7.5.1 Total lipid analysis with acid hydrolysis

Total lipid of flour and gluten was determined using AACC method 30-10 (AACC, 1995a). Flour and gluten (1 g) was placed in Mojonnier tube, mixed with 2 mL of ethanol and 10 mL of hydrochloric acid (36%) and then heated at 70°C in a water bath. When the sample was completely hydrolysed (approximately 30 min), 10 mL of ethanol was added and the tube was removed from the water bath and left to cool. Diethyl ether (25 mL) was added, followed by vigorous shaking for 1 min. Petroleum ether (25 mL) was added to the tube, again shaking vigorously for 1 min and standing until the liquid phases were well separated. The clear upper liquid (lipid extract) was transferred to the weighed round bottom flask. Lipid of the remaining liquid in the tube was re-extracted twice with 15 mL of petroleum ether. The lipid extract from two re-extractions was combined with the first extract and the solvent of the lipid extract removed by rotary evaporator at 40°C. The resultant lipid extract was further dried in an oven at 100°C for 90 min prior to cooling in a desiccator. The weight of the round bottom flask containing the dried lipid extract was recorded. A blank determination on reagents was also performed and the total lipid calculated using the following formula:

$$\text{Total lipid (\% or g/100 g of sample)} = \frac{\text{Weight of lipid (g)} - \text{weight of blank (g)}}{\text{Weight of sample (g)}} \times 100$$

7.5.2 Single solvent extraction of flour

Solvent preparation

Chloroform/methanol (1:1, v/v): Chloroform (50 mL) was mixed well with methanol (50 mL). This solvent mixture was prepared for making lipid standard solutions (Section 7.4.5) and for dissolving the ethanol or bound lipid extract from all lipid extraction procedures (Sections 7.4.3 and 7.4.4).

Extraction procedure

The single solvent extraction was performed in triplicate with petroleum ether, chloroform or ethanol. Solvent (200 mL) was added to flour (100 g) and mixed at room temperature (approximately 20°C) for 15 min using a magnetic stirrer (MacRitchie, 1985). The slurry was filtered with a Buchner funnel using a Whatman No 45 paper. The extract was collected and refiltered using normal filtration and a Whatman No 1 paper. The solvent in the lipid extracts was removed using a rotary evaporator at 40°C for the petroleum ether fraction, 60°C for chloroform fractions and 75°C for ethanol fractions. The lipid extract was further dried under a nitrogen stream and the weight recorded. The amount of lipid extract from 100 g of flour or percentage of extractable lipid in flour is calculated as follows:

$$\% \text{ extractable lipid} = \frac{\text{Amount of lipid extract (g)}}{\text{Amount of flour (g)}} \times 100$$

After recording the weight, the lipid extract was diluted with chloroform and stored at −20°C prior to further analysis.

7.5.3 Lipid extraction procedure for flour and gluten

Lipids were extracted from samples using a sequential solvent extraction, in which petroleum ether was used for the first solvent fraction, chloroform for the second and ethanol for the third (Figure 7.4). A flour sample (100g) or gluten sample (25 g) was

mixed with solvent in the ratio of 1:2 (w/v) for 15 min (MacRitchie, 1985). The slurry was filtered with a Buchner funnel using a Whatman No 45 paper and refined by normal filtration using a Whatman No 1 paper. For gluten samples, hot ethanol (70°C) was used to facilitate lipid extraction.

The solvent in the lipid extracts was removed using a rotatory evaporator at the temperature appropriate to the solvent (Section 7.4.1) and the lipid extract further dried under a nitrogen stream. Lipids were re-dissolved with chloroform for petroleum-extracted lipids and with chloroform / methanol mixture (1:1, v/v) for chloroform- and ethanol-extracted lipids prior to storage at –20°C.

7.5.4 Lipid extraction procedure for gluten control and fractions of the single and sequential acetic acid fractionations

The lipid extraction procedure for petroleum ether defatted gluten and its fractions was established based on the procedure described in Section 7.4.3 with some modifications. Lipid was extracted twice with each solvent: firstly with petroleum ether followed by hot ethanol (70°C) to extract free lipid and bound lipid, respectively (Figure 7.5). The extraction was performed at the ratio of 1:20 (w/v). The solvent was removed using a rotatory evaporator at 40°C for free lipid extract and at 75°C for bound lipid extract and then flushing with a nitrogen stream to completely remove all remaining solvent. The free and bound lipid extracts were dissolved in chloroform and chloroform/methanol (1:1, v/v) respectively, at a concentration of 10 mg/mL and stored at –20°C.

7.5.5 Lipid analysis using HPLC

Eluent solution preparation

Solvent A: N-ethyl morpholine (1 mL) and glacial acetic acid (225 μL) are dissolved in isopropanol (10 mL) and then diluted to 500 mL using 2,2,4 trimethyl pentane.

Solvent B: Dichloromethane

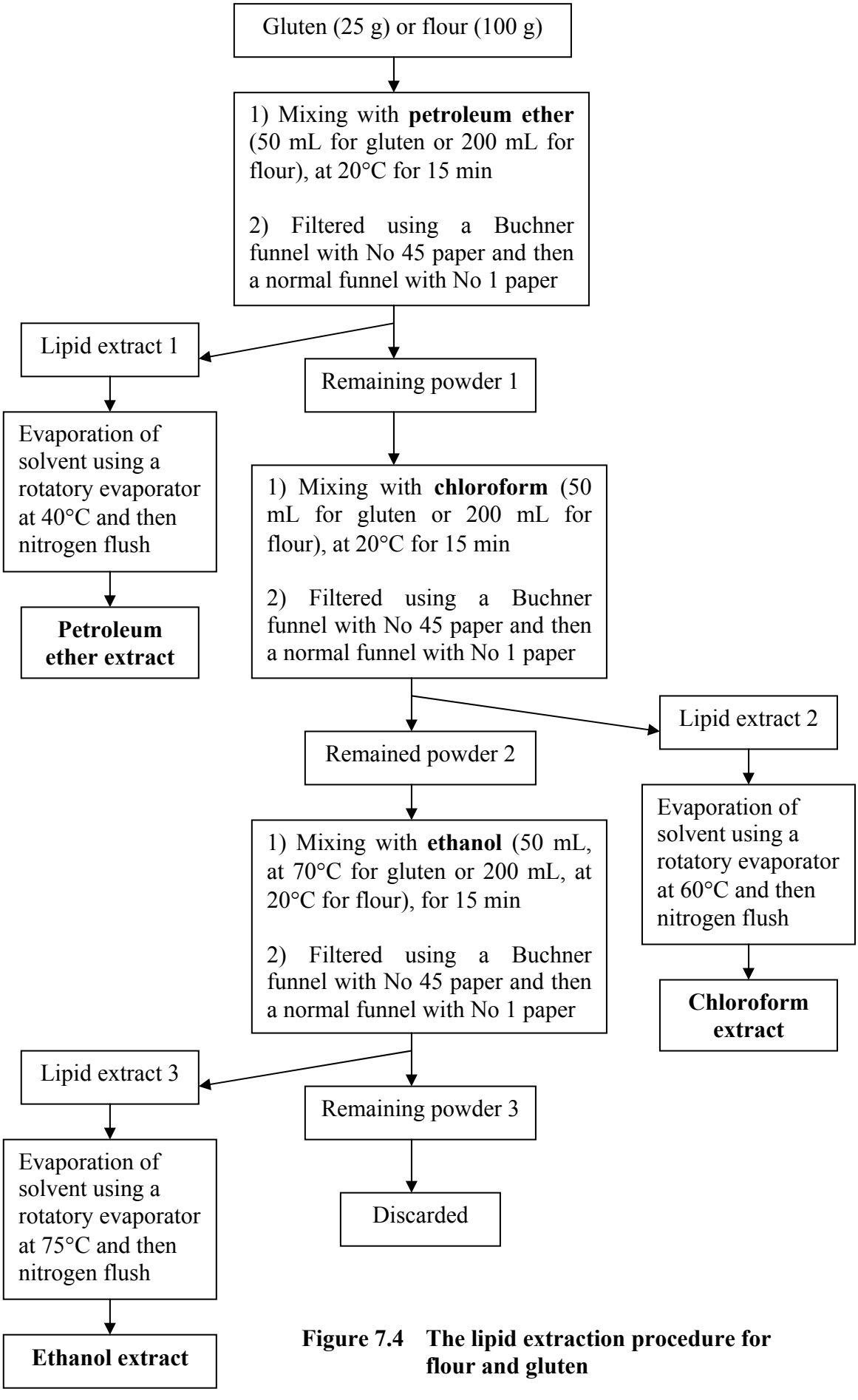


Figure 7.4 The lipid extraction procedure for flour and gluten

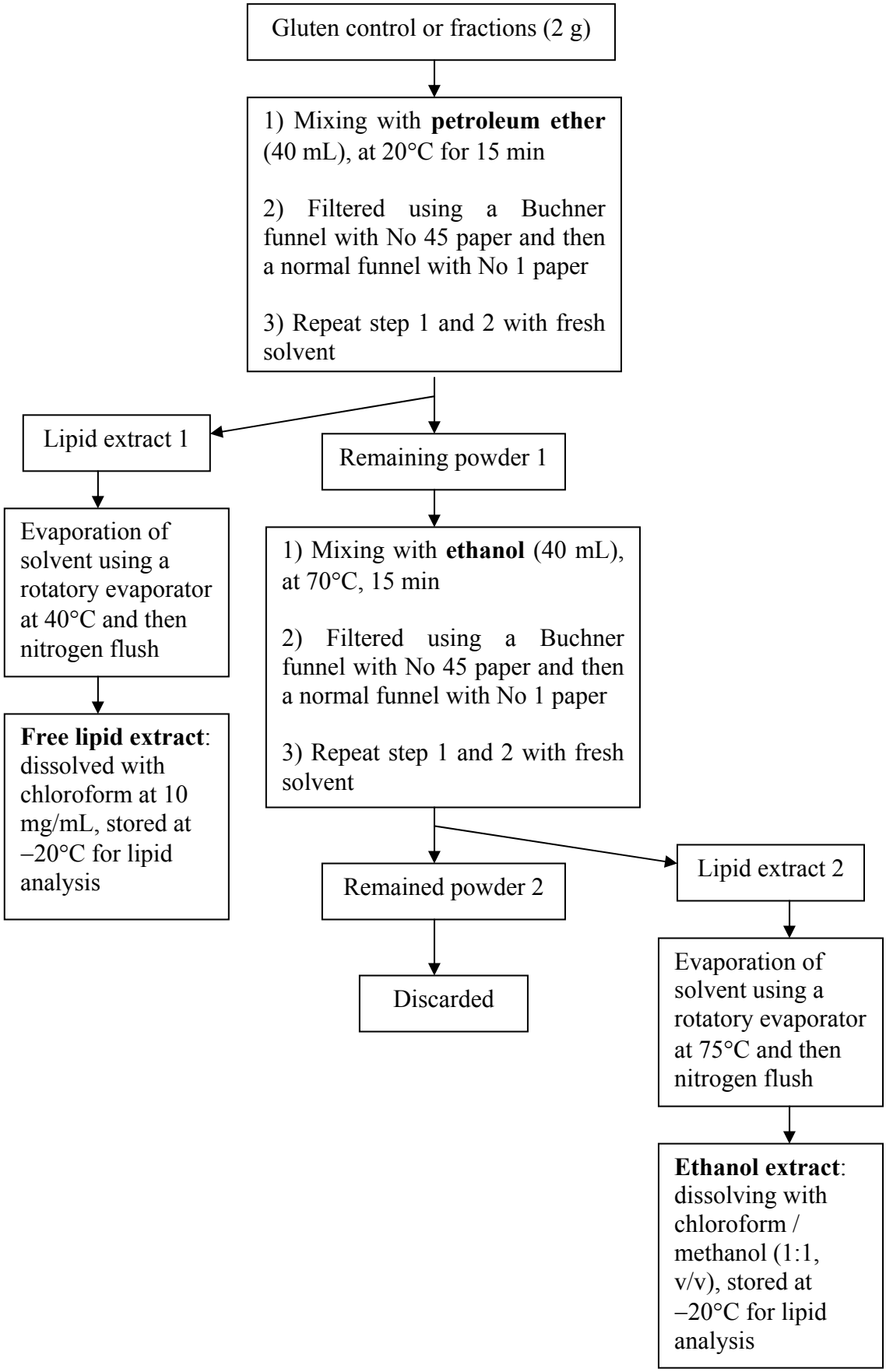


Figure 7.5 Lipid extraction procedures for gluten control and fractions of the single and sequential acetic acid fractionations

Solvent C: N-ethyl morpholine (1 mL) and glacial acetic acid (450 μL) were diluted to 500 mL with methanol.

Solvent D: Methanol

All of eluent solutions were freshly made weekly and filtered prior to the analysis.

Lipid standard preparation

Lipid standard solution (1 mg/mL): Lipid standard (10 mg) was precisely weighed and then diluted to 10 mL with solvent depending on the type of lipid. Chloroform was used for glyceryl trilinoleate, 1,3-dilinoleoyl-rac-glycerol and 1-linoleoyl-rac-glycerol. A mixture of chloroform/methanol (1:1, v/v) was used for the rest of lipid standards. All of lipid standard solutions were stored at −20°C.

HPLC procedure for lipid analysis on PVA Sil column

Lipid extract were separated using HP1100 module HPLC connected with a polyvinyl alcohol chemically bonded stationary phase PVA Sil column (5 μm, 250 mm × 4.6 mm; YMC, Japan), eluted at flow rate of 1 mL with a gradient solvent system (Table 7.9) containing 2,2,4 trimethyl pentane, isopropanol, dichloromethane, methanol, N-ethyl morpholine and glacial acetic acid (Fagan et al., 2004). Lipid compounds were detected using PL-ELS 1000 detector operated at 40°C for nebulization and at 80°C for evaporation with 1.0 mL/min of gas flow.

Calculation of relative amount of lipid classes determined by HPLC

The amounts of lipid classes are calculated as the relative amount of lipid in lipid extract. For all samples, the concentration of lipid and injection volume was kept constant. Despite this, the area of each lipid class was seen to vary. This is due to the difference in sensitivity of the detector to the different lipid classes. In order to avoid the variation of the sensitivity of lipid, the area factor of each of lipid class was calculated using the area of the lipid standard. The area factor of LPC is chosen as 1 because its

area is relatively low compared to other lipid classes. The area factors of lipid classes are calculated as follows:

$$\text{Area factor} = \frac{\text{Area of lipid class*}}{\text{Area of LPC*}}$$

* Area of lipid injected at 20 μL, 0.5 mg/mL

Table 7.9 Gradient eluent system used for separation of lipid classes

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)	Solvent D (%)
0	40	60	0	0
3	40	60	0	0
18	10	60	30	0
28	0	40	60	0
28.1	0	40	0	60
31	0	20	0	80
33	0	100	0	0
35	40	60	0	0
40	40	60	0	0

Notes: Solvent A 2,2,4 trimethyl pentane / isopropanol / N-ethyl morpholine / glacial acetic acid (98:2:0.2:0.045, v:v)
Solvent B Dichloromethane
Solvent C Methanol / N-ethyl morpholine / glacial acetic acid (100:0.2:0.09, v:v)
Solvent D Methanol

A series of lipid standards were run repeatedly to determine the area factor of each lipid class for every HPLC analysis. The amount of lipid class in lipid extract was calculated as below:

$$\text{Lipid class (\%, w/w)} = \frac{\text{Area of lipid class}}{\text{Area factor}} \times \frac{1}{\text{Sum of area of all lipid classes after adjusted with area factor}} \times \text{Lipid extract (\%, w/w)}$$

7.5.6 Lipid analysis using TLC

Lipid extract were separated on the TLC plate coated with silica gel 60 F_{254} (20 × 20 cm) using the method reported by (McCormack et al., 1991).

Solution preparation

Chloroform-methanol-water (90:20:2, v/v): Chloroform (180 mL), methanol (40 mL) and deionised water (4 mL) were thoroughly mixed.

10% (v/v) sulfuric acid in ethanol: Concentrated sulfuric acid (10 mL) was added to ethanol (90 mL) and mixed well.

Lipid standard solutions: refer to Section 7.4.5

Procedure for lipid analysis using TLC

Conditioning the TLC plate coated with silica gel 60 F_{254} (20 × 20 cm): The chloroform-methanol-water solution was added to the TLC tank to give an approximate depth 1 cm. A piece of filter paper (square 20 × 20 cm) was dipped into the solution and sufficient solution was added to allow the paper adhere to one of the inner walls of the tank. After sealing with the lid, the tank was allowed to equilibrate for at least 15 min. After marking the sample lane (1.5 cm from the bottom) and the end of solvent line (1 cm from the top) on the TLC plate using a pencil, the plate was placed into the tank until solvent reached the marked solvent line (approximately 2 hrs). The TLC plate was taken out of the tank, air-dry in the fume hood overnight and then activated in an oven at 120°C for 30 min. The conditioned TLC plate was placed horizontally in a desiccator for cooling.

Separating lipid samples on the TLC plate: Lipid standards and samples were spotted on the sample lane at 1.5 cm intervals and at a volume of 5–30 μL to give approximately 0.04 mg of lipid. After all spots of lipid standard and samples were dried, the TLC plate was placed gently into the solvent tank and then the lid was closed. The separation was performed until the solvent reached the marked solvent line, approximately 1 hr. The

TLC plate was air-dried in the fume hood for 30 min before being gently sprayed with the 10% sulfuric acid in ethanol from the top to the bottom. After air-drying the plate again, the lipid spots were visualised by charring the plate in an oven at 160°C for 15 min.

7.6 General procedure for protein analysis in flour, gluten and gluten fractions

7.6.1 Gluten protein analysis using SDS-PAGE

Proteins of gluten and gluten fractions were analysed using the SDS-PAGE method described by (Kasarda et al., 1998). This was performed using the Novex Xcell Mini cell with a NuPAGE gradient gel (4-12%) Bis-Tris [Bis (2-hydroxyethyl) imino (hydroxymethyl) methane-HCl] and Novex MES [2-(N-morpholino) ethane sulfonic acid] running buffer.

Solution preparation for SDS-PAGE

Dithiothreitol 0.5 M (stock solution): Dithiothreitol (1.542 g) was dissolved in 10 mL of Milli Q water and then diluted to 20 mL. This solution was aliquot at 1 mL into a 1.5 mL Eppendorf tube and stored at –20°C for subsequent use in preparing a sample buffer of a reduced SDS-PAGE and a protein solubilisation buffer for two-dimensional electrophoresis (Section 7.6.2).

MES [2-(N-morpholino) ethane sulfonic acid] buffer solution: MES buffer consisted of 50 mM MES, 50 mM Trizma base, 0.1% SDS, 1 mM EDTA, pH 7.3. The 20 × MES running buffer was prepared by dissolving MES.H_2O (106.63 g), Trizma base (60.6 g), SDS (10 g) and EDTA (3 g) in 400 mL of Milli Q water, mixing well, adjusting pH to 7.3 with hydrochloride acid (32%) and diluted to 500 mL. The 20 × MES running buffer can be stored at 4°C for 6 months. For electrophoresis, MES running buffer was freshly prepared by diluting 20 × MES running buffer (50 mL) to 1,000 mL with Milli Q water.

Sample buffer solution: The reduced SDS-PAGE sample buffer consisted of 3.6% SDS, 18% glycerol, 50 mM dithiothreitol, 112.3 mM Tris (hydroxymethyl) amino methane and bromophenol blue as marker dye (0.0045%), pH 8.5. As dithiothreitol must be added into the sample buffer just prior to the analysis, a sample buffer without

dithithreitol (solution A) was prepared by dissolving SDS (4 g), glycerol (20 g), Tris base (1.512 g) and bromophenol blue (0.005 g) into 70 mL of Milli Q water, adjusting pH to 8.5 using concentrated hydrochloric acid (32%) and diluted to 100 mL. Solution A can be stored at 4°C for 6 months. In order to prepare a sample buffer for the reduced SDS-PAGE, the solution A (9 mL) was mixed well with dithiothreitol (0.5 M, 1 mL). With the non-reduced SDS-PAGE, the sample buffer was prepared by mixing the solution A (9 mL) and Milli Q water (1 mL).

Coomassie Brillianet Blue R-250 staining solution (0.1% of Coomassie Blue R-250 in 40% methanol and 10% acetic acid): Staining solution was prepared by dissolving of Coomassie R-250 (1.0 g) in 1,000 mL of the solution containing 40% methanol and 10% acetic acid. This solution was made by mixing methanol (400 mL) with acetic acid (100 mL) and then diluted to 1,000 mL using Milli Q water.

Destaining solution (10% ethanol and 7.5% acetic acid): A destaining solution was prepared by mixing methanol (100 mL) with acetic acid (75 mL) and then diluted to 1,000 mL using Milli Q water.

Procedure of SDS-PAGE for gluten protein analysis

Sample preparation for SDS-PAGE: Flour (20 mg) or gluten (10 mg) samples were mixed with the sample buffer (1 mL) in Eppendorf tube using a vortex for 1 hrs. These samples were heated at 85°C for 10 min to facilitate protein extraction.

SDS-PAGE performance: Sample was loaded at 30 μg of proteins per slot. The Novex Mark 12 unstained standard (5 μL) was included on gels. Antioxidant (0.5 mL) was added into the running buffer chamber when running a reduced sample. The electrophoresis conditions are described in Table 7.10 for reduced and non-reduced samples.

Coomassie staining and de-staining procedure: During staining and de-staining procedure, gels were shaken on an orbital shaker. After electrophoresis, the gel was placed in 100 mL of Coomassie Brilliant Blue R-250 staining solution and shaken for 25 min. The staining solution was discarded and the de-staining procedure was

performed with three changes of de-staining solution. Firstly, the de-staining solution (100 mL) was added and shaken for 30 min for the stain solubilised in the solution, which was then removed. Secondly, fresh de-staining solution (100 mL) was added then gently heated using a microwave for 30 sec and shaken for another 1 hr. After removing the solution, more fresh de-staining solution (100 mL) was added and shaken overnight. The de-staining procedure was considered complete when the background of the gel was clear and the gel was then photographed.

Table 7.10 SDS-PAGE conditions for reduced and non-reduced samples

Step	Reduced SDS-PAGE		Non-reduced SDS-PAGE	
	Voltage constant (V)	Time (min)	Voltage constant (V)	Time (min)
1	100	60	80	75
2	120	50	90	15
3			95	60

7.6.2 Protein analysis using a two-dimension electrophoresis

The two-dimensional electrophoresis procedure was based on the method described by Skylas et al. (2000) with modification to suite a small gel (8 cm × 8 cm). Isoelectric-focusing (IEF) was performed using ZOOM IPG Runner (Invitrogen, USA). The second dimension was performed with a NuPAGE gradient gel (4-12%) Bis-Tris [Bis (2-hydroxyethyl) imino (hydroxymethyl) methane-HCl] and Novex MES [2-(N-morpholino) ethane sulfonic acid] running buffer in the Novex Xcell Mini cell.

Solution preparation for two-dimension electrophoresis (2-DE)

Bromophenol blue 0.1% (stock solution): Bromophenol blue (0.010 g) was dissolved in 5 mL of Milli Q water and then diluted to 10 mL. This solution was used to prepare a protein solubilisation buffer.

1.5 M Tris /HCl pH 8.8 (stock solution): Tris base (18.171 g) was mixed with 80 mL Milli Q water, adjusted pH to 8.8 using hydrochloric acid (32%) and then diluted to 100 mL. This solution was used to prepare an equilibration buffer solution.

Protein solubilisation buffer solution: Protein solubilisation buffer solution consisting of 7 M urea, 2 M thiourea, 4% CHAPS, 1% Ampholytes, 40 mM Tris, 0.002% Bromophenol Blue and 50 mM dithiothreitol was prepared by dissolving urea (10.51 g), thiourea (3.81 g), CHAPS (1.0 g), Tris base (0.121 g), ampholytes (250 μL), and 0.5 mL of bromophenol blue 0.1% in 15 mL of Milli Q water and then diluted to 25 mL. Aliquot of buffer (0.9 mL) was transfered into 1.5 mL Eppendorf tubes and stored at –20°C. Dithiothreitol (0.5 M, 0.1 mL, prepared in Section 7.6.1) is added into the 0.9 mL buffer and mixed well just prior to analysis.

Equilibration buffer solution: contained 6 M urea, 2% sodium dodecyl sulfate, 0.375 M Tris / HCl pH 8.8, 20% glycerol, 50 mM dithiothreitol and 2.5% acrylamide. It was prepared by dissolving urea (36.04 g), SDS (2.0 g), Tris / HCl 1.5 M, pH 8.8 (25 mL), glycerol (20 g), dithiothreitol (0.771 g) and acrylamide solution 40% (6.25 mL) in 40 mL of water and then diluted to 100 mL. The prepared equilibration buffer solution was stored at –20°C.

Agarose solution (0.5%): Agarose (0.5 g) was added to 100 mL of MES buffer solution (prepared in Section 7.6.1) and heated until well dissolve (approximately 3 min) using microwave. The agarose solution was then cooled and stored at 4°C.

Isoelectric-focussing performance

Sample preparation using ZOOM 2D Protein Solubilizer: gluten sample or fraction sample (approximately 10 mg) was mixed with the protein solubilisation buffer solution (1 mL) in 1.5 mL Eppendorf tube for 2 hrs using a vortex mixer and then centrifuged at 15,000 × g for 5 min. The solubilised protein sample (supernatant) was collected and stored at –70°C until further analysis.

Loading of protein samples and re-hydration of IPG strip: A solubilised protein sample was diluted with protein solubilisation buffer solution so that the concentration of

protein was 200 μg/155 μL/strip. The diluted solubilised protein sample (155 μL) was loaded onto the sample-loading well of the ZOOM IPGRunner cassette, then the IPG strip (7 cm) was inserted for re-hydrating with protein solution overnight.

Isoelectric focussing conditions: The ZOOM IPGRunner cassette was assembled in the ZOOM IPG Runner core and Milli Q water was placed into the outer chamber. The focussing was performed for 7.5 hrs using the program specified in Table 7.11. After the focussing was completed, the IPG strips were removed from the IPG tray, placed separately in the 10 mL plastic container (15 × 98 mm) and stored at −20°C until running of the second dimension (SDS-PAGE).

Equilibrate and alkylate the IPG trip for second dimension SDS-PAGE: Before performing the second dimension SDS-PAGE, the focussed IPG strip was equilibrated and alkylated in the equilibration buffer solution (10 mL), which carried out by horizontally shaking at a room temperature for 30 min.

Table 7.11 A protocol of electro-focusing program for 4 IPG strips

Step	Voltage constant (V)	Time (hrs)	Cumulative (Vh)	Limit current (mA)	Limit power (W)
Step 1	100	1.0	100	2	2
Step 2	200	1.0	200	2	2
Step 3	300	0.5	150	2	2
Step 4	600	0.5	300	2	2
Step 5	1,000	0.5	500	2	2
Step 6	3,000	4.0	12,000	2	2
Total			13,250		

Second dimension (SDS-PAGE): Agarose solution was melt by warming using microwave. After the equilibration and alkylation process completed, the IPG strip was inserted into the well on the top of the NuPAGE 4-12% Bis-Tris ZOOM gel (8 × 8 cm). The warm agarose solution was poured over the IPG strip to form the connection with

the NuPAGE IPG gel. The second dimension (SDS-PAGE) was performed in the reduced conditions specified in Section 7.6.1.

7.6.3 NativePAGE for lipoprotein detection

A method for detection of lipoproteins in wheat protein was developed using NativePAGE Novex Bis-Tris Gel system. In this method, Coomassie G-250 was used as a charge shift molecule to bind to protein thereby conferring a net negative charge while maintaining the proteins in their native state. In this study, proteins separated on the NativePAGE Novex 3-12% Bis-Tris and lipoproteins were visualised by protein double staining using protein stain and lipid stain. The protein staining procedure was adapted from that described for the NativePAGE Novex Bis-Tris Gel system (Invitrogen, Melbourne, Australia). The lipid staining procedure was adapted from the manual of the lipoprotein electrophoresis system (Beckman Coulter, USA).

Solution preparation

20 × Running buffer: A 20 × running buffer consisting of 1 M Bis-Tris and 1 M Triscine, pH 6.8 was prepared by mixing Bis-Tris (52.3 g) and Tricine (44.8 g) with 200 mL of Milli Q water, adjusted pH to 6.8 using concentrated hydrochloric acid (32%), enabling the complete dissolution of all chemicals and made up a volume to 250 mL. This solution can be kept at room temperature for 6 months.

Anode buffer: consisted of 50 mM Bis-Tris and 50 mM Triscine, pH 6.8 was prepared by diluting the 20 × running buffer (50 mL) to 1,000 mL. This buffer was used for the outer buffer chamber and freshly made in every experiment.

Cathode buffer additive: This contained 0.4% Coomassie G-250 was prepared by dissolving Coomassie (0.10 g) in Milli Q water (15 mL) and then diluted to 25 mL.

Cathode buffer: This consisted of 50 mM Bis-Tris, 50 mM Triscine, 0.002% Coomassie, pH 6.8 was made by diluting the mixture of the 20 × running buffer (10 mL) and the cathode buffer additive (1 mL) to 200 mL. This buffer was used for the inner buffer chamber and was freshly made for each experiment.

2 × Sample buffer without salt: This solution contained 100 mM Bis-Tris, 20% glycerol (w/v), 0.004% bromophenol blue was prepared by dissolving Bis-Tris (2.092 g) with Milli Q water (50 mL), adjusted pH to 7.2 using concentrated hydrochloric acid (32%), then added glycerol (20 g) and bromophenol blue 1% (2 mL, Section 7.6.2), mixed well, and diluted to 100 mL.

2 × Sample buffer with salt: This solution contained 100 mM Bis-Tris, 100 mM NaCl, 20% glycerol (w/v), 0.004% bromophenol blue was prepared by dissolving Bis-Tris (2.092 g) and NaCl (0.0585 g) in Milli Q water (50 mL), adjusted pH to 7.2 using concentrated hydrochloric acid (32%), then added glycerol (20 g) and bromophenol blue 1% (2 mL, Section 7.6.2), mixed well and diluted to 100 mL.

Protein staining solution: This solution was prepared by dissolving Coomassie G-250 (0.2 g) in the solution of 40% methanol and 10% acetic acid (1,000 mL). The solution of 40% methanol and 10% acetic acid was made by mixing methanol (400 mL) with acetic acid (100 mL) and diluted to 1,000 mL using Milli Q water.

Protein destaining solution (10% ethanol and 7.5% acetic acid): A destaining solution was prepared by mixing methanol (100 mL) with acetic acid (75 mL) and diluted to 1,000 mL using Milli Q water.

Sudan black 7% on ethanol 95% (stock solution): Sudan Black (1.4 g) was mixed in ethanol 95% (20 mL) overnight for completely dissolving.

Working staining solution (0.07% Sudan Black in 52% ethanol): The working staining solution was freshly prepared by mixing Sudan black 7% solution (3 mL) with ethanol (95%, 162 mL) and Milli Q water (135 mL).

Lipid destaining solution (42.75% ethanol): This solution was prepared by mixing ethanol (95%, 450 mL) with Milli Q water (550 mL).

Procedure of native NuPAGE for lipoprotein analysis

Sample preparation: Gluten fraction sample was dissolved in different solutions consisting of sample buffers with and without salt and sample buffer containing dithiothreitol with and without salt. The detail of various sample buffers is presented in Table 7.12. Sample buffer was cooled to 4°C in a refrigerator overnight. Protein fraction sample (approximately 5 mg) was mixed with 1 mL of sample buffer (30 min, 4°C) using a vortex mixer and centrifuged at 16,000 × g for 5 min. The supernatants were collected and kept in an ice-bath throughout the analysis to avoid a hydrolysis of protein due to proteinases.

Native NuPAGE operation: Protein sample was loaded in 5 μL per well (approximately 20 μg of proteins). NativeMark unstained protein standard (5 μL) and lipoprotein standard (5 μL) was also loaded on gels. After assembling the gel cassette in the Novex Xcell, the cathode buffer was filled into the inner buffer chamber and the anode buffer was filled to two-thirds of the outer buffer chamber. The NativePAGE was performed at a constant voltage of 120 V for 2 hrs and then 140 V for another 2 hrs.

Table 7.12 Details of various sample buffers

	Sample buffers			
Solutions	**Without salt**	**With salt**	**Containing dithiothreitol without salt**	**Containing dithiothreitol with salt**
	(mL)	(mL)	(mL)	(mL)
Sample buffer without salt	0.5	0.0	0.5	0.0
Sample buffer with salt	0.0	0.5	0.0	0.5
Dithiothreitol 0.5 M*	0.0	0.0	0.1	0.1
Deionised water	0.5	0.5	0.4	0.4

Note: * Detail in Section 7.6.1

Protein staining and destaining procedures: After electrophoresis, the gels was stained and destained using the Coomassie staining and destaining procedures described in Section 7.6.1 but the staining time was reduced to 5 min.

Lipid staining and destaining procedures: After completing the protein staining, a lipid staining process was performed by shaking the gel in the working staining solution (100 mL) for 5 min and followed by shaking three times in fresh destaining solution (100 mL) for 5 min each time. After discarding the last lipid destaining solution, the gel was placed in the protein destaining solution (100 mL) for 1 hr to restore the shape of the gel which was then ready to be photographed.

7.6.4 Size-exclusion high-performance liquid chromatography

Solution preparation

Disodium hydrogen othorphosphate (Na_2HPO_4) 0.05 M solution: a $Na_2HPO_4.2H_2O$ (1.780 g) was dissolved in Milli Q water (200 mL).

Sodium dihydrogen othorphosphate (NaH_2PO_4) 0.05 M solution: a $NaH_2PO_4.2H_2O$ (1.560 g) was dissolved in Milli Q water (200 mL).

Phosphate buffer 0.05 M solution: The NaH_2PO_4 0.05 M solution (55 mL) was mixed the Na_2HPO_4 0.05 M solution (100 mL). The pH was adjusted to 6.9 using either NaH_2PO_4 (0.05 M) for decreasing pH or Na_2HPO_4 (0.05 M) for increasing pH.

Sample buffer (0.05 M phosphate buffer, pH 6.9 containing 0.5% SDS): prepared by dissolving SDS (0.5 g) in phosphate buffer (0.05 M, 100 mL).

Eluent solution (acetonitrile- water, 1:1, v/v containing 0.1% trifluoroacetic acid): acetonitrile (500 mL) and trifluoroacetic acid (1 mL) was mixed and diluted to 1,000 mL using Milli Q water.

SE-HPLC procedure for analysis of protein in gluten and gluten fractions

The protein compositions of gluten and gluten fractions were analysed using SE-HPLC method (Batey et al 1991). The sample (10 mg) was extracted with 0.05 M phosphate buffer, pH 6.9 containing 0.5% SDS (1 mL) for 60 min using a vortex followed by sonication using Bransonic 221 (Branson, Shelton, USA) at 50/60 Hz for 10 min. The protein extract was centrifuged at 15,000 × *g* for 5 min and filtered through a 0.45 μm filter (Bonnet, NSW, Australia). The protein extract (20 μL) was injected onto the Biosep-SEC S4000 column (Phenomenex, NSW, Australia), connected to a Shimadzu HPLC system (Shimadzu, Japan) consisting of LC-10A model pumps, SIL-20A automatic sampler and a model SPD-20A UV-visible detector. Samples were eluted under isocratic conditions using a solvent containing 0.1% trifluoroacetic acid in 50% acetonitrile at a flow rate of 0.5 mL/min and monitored by UV detection at 214 nm.

7.6.5 Reversed-phase high-performance liquid chromatography

Solution preparation

Tris 1 M solution: Tris base (0.605 g) was dissolved in Milli Q water and diluted to 10 mL.

Guanidine solution 6.67 M, pH 8: Guanidine (63.7 g) was dissolved in Milli Q water, adjust pH to 8 using Tris 1 M solution and then diluted to 100 mL.

Sample buffer (Guanidine HCl 6 M containing 50 mM dithiothreitol): A 6.67 M guanidine solution pH 8 (0.9 mL) was mixed well with the 0.5 M dithiothreitol (0.1 mL). This solution was prepared on the day of analysis.

Acetonitrile containing 0.05% trifluoroacetic acid: Trifluoroacetic acid (0.5 mL) was diluted to 1,000 mL using acetonitrile.

Milli Q water containing 0.05% trifluoroacetic acid: Trifluoroacetic acid (0.5 mL) was diluted to 1,000 mL using Milli Q water.

RP-HPLC procedure for analysing protein in gluten fractions

Proteins in gluten fractions were separated using the method reported by (DuPont et al., 2005). Gluten fraction was dissolved at a concentration of 3 mg of protein/mL in 6 M guanidine HCl containing 50 mM dithiothreitol. Protein solution was centrifuged at 15,000 × g for 5 min and was filtered through a 0.45 μm filter. Protein extract was injected onto a Jupiter C18 semi-preparative RP-HPLC (5 μm, 300 Å, 250 × 4.6 mm) (Phenomenex, NSW, Australia) connected to a Shimadzu HPLC system (Shimadzu, Japan) consisted of LC-10A model pumps, SIL-20A automatic sampler and a model SPD-20A UV-visible detector, at a volume of 20 μL for analytical purposes and 100 μL for collection of the fractions. The column was heated to 50°C. Proteins were eluted using a gradient of 10–65% acetonitrile in 0.05% trifluoroacetic acid for 60 min at flow rate of 1.0 mL/min. For fraction collection, the eluent from the column was connected to the fraction collector (FRC-10A, Shimadzu, Japan). The fraction was set up to collect in 1 min intervals from 25 min to 54 min, giving a total of 29 fractions.

7.6.6 Capillary electrophoresis for TX114 protein extract

Solution preparation

NaOH 1 M: NaOH (40 g) was dissolved in 80 mL of deionised water and diluted to 100 mL.
NaOH 0.1 M: A 1 M NaOH solution (10 mL) was diluted to 100 mL using deionised water.

Phosphoric acid 1 M: Concentrated phosphoric acid (85%,10 mL) was mixed well with 140 mL of water.

Phosphate buffer 100 mM, pH 2.5 containing 0.05% HPMC: $NaH_2PO_4.2H_2O$ (10.01 g) was dissolved in approximately 900 mL of deionised water, added concentrated phosphoric acid (85%, 1.84 mL) and mixed well. HPMC (0.50 g) was added to this solution, stirred overnight at a room temperature and then diluted to 1,000 mL using deionised water. The pH was recorded but not adjusted. The buffer was freshly prepared each week and degassed for 1 hr using vacuum system prior to the analysis.

Capillary electrophoresis procedure for TX114 protein extract

Proteins from TX114 extracts were analysed on CE using the method reported previously (Day and Schofield, 2001). The analysis was performed on the capillary electrophoresis system (P/ACE MDQ, Beckman Coulter, USA), using the uncoated fused silica capillary column (40 cm × 50 μm) that was conditioned with the program in Table 7.13. The running program was described in Table 7.14. Protein extract was injected into the conditioned silica capillary using a pressure of 1.5 psi for 20 sec. The separation was performed at a voltage of 15 kV at 25°C for 25 min and protein was detected at 200 nm.

Table 7.13 The conditioning program for the uncoated silica capillary column

Event	Value	Time	Inlet vial	Outlet vial	Summary	Solution
Rinse pressure	20 psi	1 min	BI:B1	BO:B1	Forward	1 M NaOH
Rinse pressure	20 psi	1 min	BI:C1	BO:B1	Forward	0.1 M NaOH
Rinse pressure	20 psi	5 min	BI:A1	BO:B1	Forward	Water
Inject pressure	1 psi	10 sec	BI:A1	BO:B1	Override, forward	Water
Separation voltage	15 kV	10 min	BI:A1	BO:A1	0.17 min ramp Normal polarity	
End						

7.6.7 DC protein assay

Protein standard preparation

Bovine serum albumin was used as a protein standard for this assay. A stock solution of protein standard (2 mg/mL) was prepared by dissolving bovine serum albumin (100.0 mg) in deionised water (40 mL) and diluted to 50 mL. A series of protein solution for a

standard curve was prepared by diluting the prepared stock solution with deionised as described in Table 7.15. The diluted protein standards were mixed well before performing the DC protein assay. The standard curve was prepared each time the assay was performed.

Table 7.14 The running program for CE protein analysis

Event	Value	Time	Inlet vial	Outlet vial	Summary	Solution
Rinse pressure	20 psi	1 min	BI:D1	BO:B1	Forward	1 M H_3PO_4
Rinse pressure	20 psi	1 min	BI:A1	BO:B1	Forward	Water
Rinse pressure	20 psi	3 min	BI:E1	BO:B1	Forward	Running buffer
Inject pressure	1.5 psi	10 sec	BI:A1	BO:B1	Override, forward	Protein samples
Separation voltage	15 kV	25 min	BI:A2	BO:A2	0.17 min ramp Normal polarity	
End						

Table 7.15 The details of the preparation of a protein standard for DC protein assay

Concentration of protein standard series (mg/mL)	Volume of 2 mg /mL stock solution of protein standard (μL)	Volume of added deionised water (μL)
0.2	20	180
0.4	40	160
0.6	60	140
0.8	80	120
1.0	100	100
1.2	120	80
1.4	140	60

Procedure of DC protein assay

Protein assays were conducted on a microplate using chemicals provided in the DC protein assay (Bio-Rad, NSW, Australia). The aliquots of solutions in this assay was taken with a multi-channel pipette. Protein standards or samples (5 μL) were dispensed into wells. The reagent A (25 μL) was firstly added. After a gently mixing, the reagent B (200 μL) was added and then mixed thoroughly using the shaker in the microplate reader. The samples were incubated at room temperature for 15 min, and then the absorbance measured at 750 nm. The assay of protein standards and samples was performed in triplicates. Some protein samples required dilution if the absorbance was outside of the range of the standard curve.

Calculation

The protein concentration of sample was calculated based on the equation of trendline from the standard curve (y = ax + b) as below:

$$\text{Protein concentration (mg/mL)} = \frac{\text{Absorbance of sample} - b}{a} \times \text{Dilution factor}$$

7.6.8 Amino acid analysis

Amino acid composition of gluten fractions was analysed by the Australian Proteome Analysis Facility Ltd (APAF, Sydney, Australia). The samples were hydrolysed using 6 M hydrochloric acid at 110°C for 24 hrs. After hydrolysis, all amino acids were analysed using the Waters AccQTag chemistry. Cysteine and tryptophan were not analysed in this method. Samples were analysed in duplicate and results are expressed as an average (Appendix).

Chapter 8

Results and discussion: Extractability and composition of lipid in flour and gluten

8.1 Introduction

Lipids in flour have been classified into two broad categories which are non-starch lipids and starch lipids. The latter are present within starch granules while non-starch lipids are not. Non-starch lipids are available to interact with other flour components, particularly proteins and therefore are of most interest for the purpose of this study. The non-starch lipids can be further divided into free lipids and bound lipids based on their extractability. Free lipid can be extracted with solvents having relatively low polarity including petroleum ether, hexane or diethyl ether while bound lipid can only be extracted with more polar solvents such as chloroform, ethanol or a mixture of an alcohol and water (Chung and Ohm, 2000; Cornell and Hoveling, 1998b).

Typically, total lipid in flour and cereal products has been determined using the AACC 30-10 (AACC, 1995a) as a standard method. The acid used in this method causes the hydrolysis of various lipid components, so it cannot be applied to extract lipids for the quantitation of the different lipid classes. On the other hand, solvent extraction does not affect the molecular structure of lipid components when extracting lipid from flour or gluten. Accordingly, many single and mixed solvent extraction methods have been used to extract wheat lipids and these have been reviewed in Section 3.3.

Among the commonly used systems, petroleum ether or hexane has been used to extract free lipid from flour (Morrison, 1976) and more polar solvents including ethanol, 1-butanol saturated with water (WSB) or chloroform / methanol (2:1, v/v) extract both free and bound lipids. Different solvents have varying effectiveness in extracting lipids from wheat flour and gluten (Cornell and Hoveling, 1998b). The WSB extraction was the first approach recommended for estimation of lipid in wheat products (Mecham and Mohammed, 1955) because higher amounts of lipids are obtained, however, some non-lipid material is also extracted. A mixture of chloroform and methanol can be used for

extraction of lipid from dough but give lower results than either ethanol or WSB (Tsen et al., 1962). Ethanol has been reported to be very useful, as it can subsequently be removed more readily from the lipid extract than WSB (Cornell and Hoveling, 1998b).

Selective solvent extraction has been used to separately extract non-starch and starch lipid from wheat flour (Morrison et al., 1975). Recently, a selective procedure has been developed to firstly extract free lipid with petroleum ether and then bound lipid using an acid hydrolysis method (Ruibal-Mendieta et al., 2002). Hence, a sequential extraction of lipid from flour or gluten using solvents of differing polarity can be applied to separately extract free and bound lipids.

There are many different lipid classes present in both free and bound lipid extracts of flour. They can be divided into three broad classes consisting of non-polar lipids, glycolipids and phospholipids. Various methods have been developed to separate and determine the lipid classes present in wheat flour using thin-layer chromatography (TLC) (Pomeranz and Chung, 1965; Clayton et al., 1970) and, more recently, HPLC in conjunction with ELSD (Conforti et al., 1993; Néron et al., 2004). Other methods have been used to determine the content of free glycolipids in flour using a scanning spectrophotometer (Ohm and Chung, 2000) as well as FAME on gas chromatography (Morrison et al., 1980). The theory and technique of these methods as well as their advantages and disadvantages in determination of lipid classes have been discussed in Section 3.4. TLC is a qualitative method whilst FAME gas chromatography can be used for quantification but the separation of lipid class still depends on the TLC method. HPLC with ELSD has been established as an advanced method for separation and quantification of lipid classes. All lipid classes can be quantitated without prior fractionation of the individual glycolipids and phospholipids (Conforti et al., 1993) but a long running time is required, typically exceeding one hour. Another procedure (Néron et al., 2004) provides shorter running times but is limited to separating the components of the phospholipid class.

The purpose of this study has been firstly to modify the lipid analysis method using HPLC-ELSD in order to determine all lipid classes in wheat flour and gluten with a shorter running time and secondly to develop a lipid extraction method for quantifying levels of free and bound lipid in flour and gluten. The selected procedures are then used

to determine the lipid class composition of free and bound lipids in flour and gluten for evaluating the occurrence of protein and lipid interactions in gluten.

8.2 Separation and identification of lipid classes using HPLC

A series of lipid classes consisting of thirteen lipid components has been separated on the PVA silica column using the method of Fagan et al. (2004) with a modification (Figure 8.1). This method was able to separate all main lipid classes of flour lipid in a shorter time than the method previously developed by Conforti et al. (1993). Within same period of time, the current method can resolve a separation of a wider range of lipid classes compared to the previous method (Néron et al., 2004).

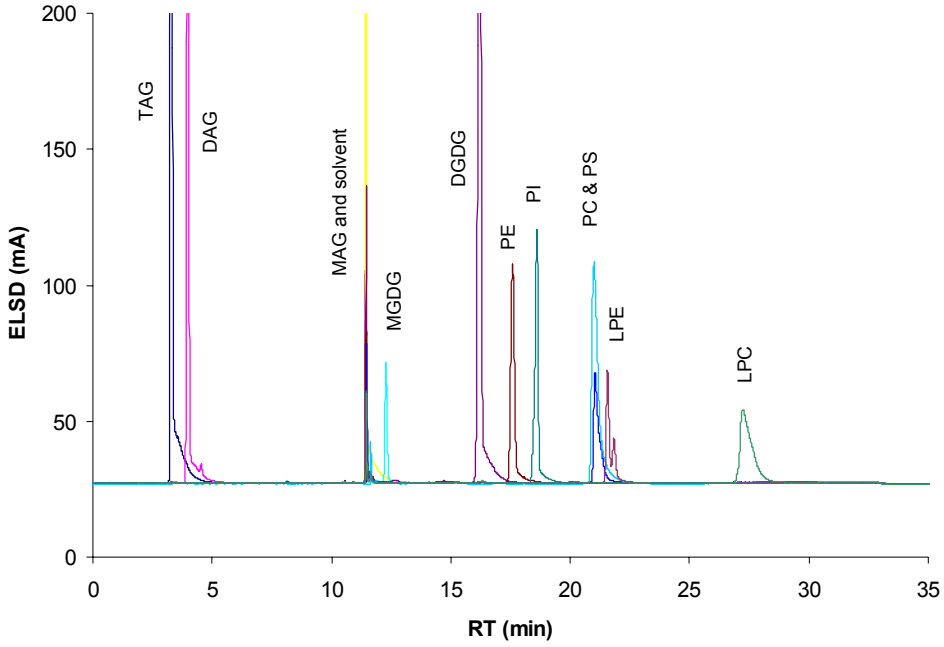


Figure 8.1 HPLC chromatogram of lipid standards

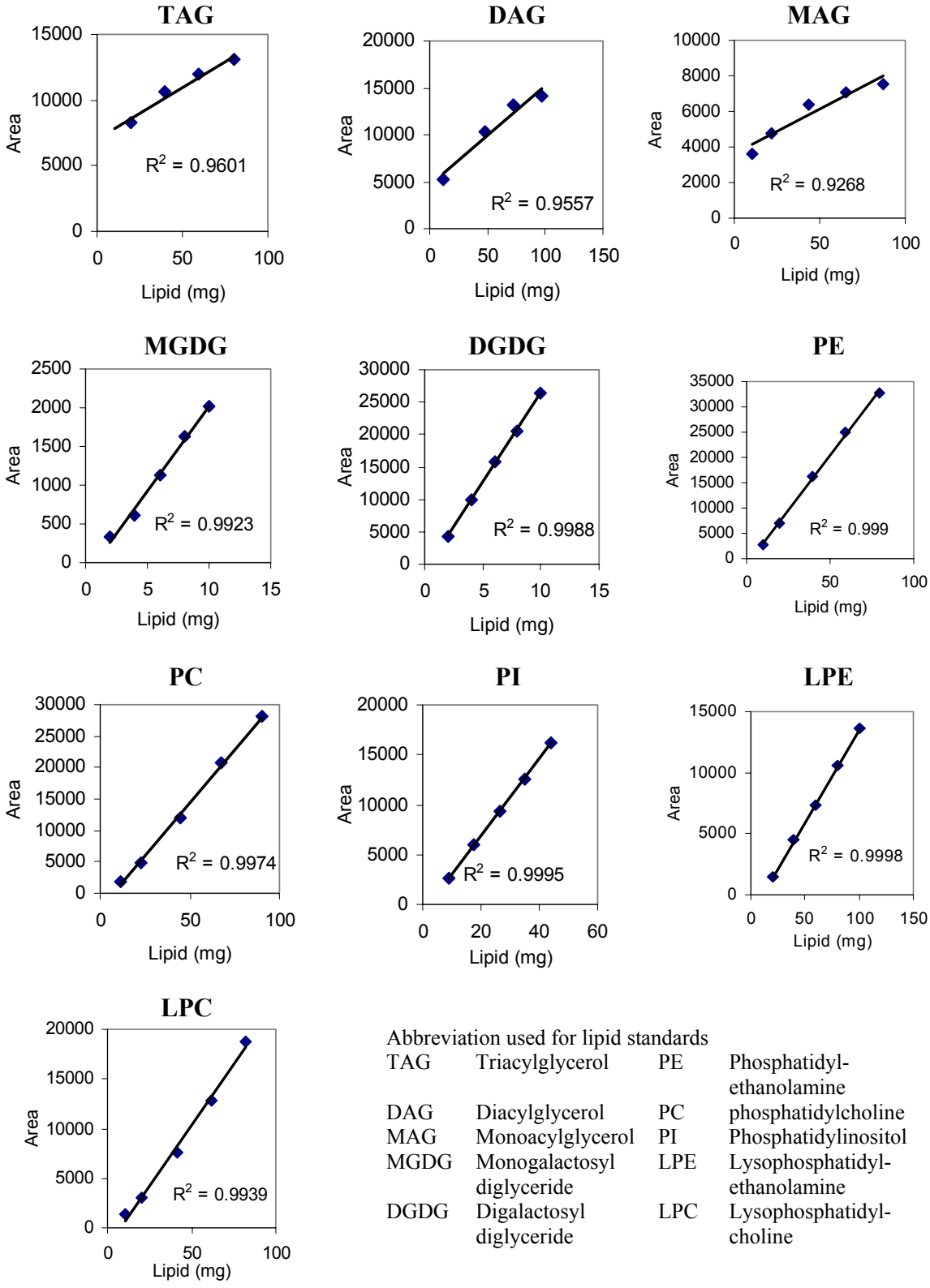
Notes:				
	TAG	Triacylglycerol	PI	Phosphatidyl inositol
	DAG	Diacylglycerol	PS	Phosphatidyl serine
	MAG	Monoacylglycerol	PC	Phosphatidyl choline
	MDDG	Monogalactosyl diglyceride	LPE	Lysophosphatidyl ethanolamine
	DGDG	Digalactosyl diglyceride	LPC	Lysophosphatidyl choline
	PE	Phosphatidyl ethanolamine		

Lipid classes in flour lipid extract were identified by a comparison of retention time of lipid classes in the sample to the known lipid in lipid standards. The retention time can be slightly shifted due to the lifetime of the HPLC column. Therefore, the series of lipid standards were always re-run each time of the HPLC analysis. As limitation of ELSD, the peak area and lipid mass is linear in a certain range of lipid concentrations (Moreau, 1994). The standard curve was established for each lipid class in the range of working concentrations (Figure 8.2). All lipid standard curves are linear with the square values ranged from 0.93 to 0.99

8.3 Single solvent extraction of flour

Commercial flour (S008) was used to select the extraction method for the study. Total lipid of flour as a reference value was determined using the acid hydrolysis method (AACC, 1995a). For a single solvent extraction, a series of three solvents were trialled and the flour was extracted repeatedly (three times) at room temperature for each solvent. Petroleum ether, chloroform and ethanol were chosen for this study as they have been widely applied as solvents for lipid extraction and have differing polarities.

The total amount of lipid extracted using either petroleum ether, chloroform or ethanol was lower than that determined using the acid hydrolysis method (Table 8.1). The results for lipid extracted with petroleum ether and chloroform were in the range of lipid contents reported for Australian wheat flours by McCormack et al. (1991). The relatively low levels of total extractable lipid resulting from solvent extraction may reflect the varying extractability of flour lipid components in solvents of different polarities. Alternatively, some of the lipid extracted at the higher temperatures involved in the acid hydrolysis procedure may be lipids found within starch granules. As the acid hydrolysis procedure will cause swelling and gelatinisation of starch granules, it allows starch lipid to be removed by solvent; therefore, the total lipid determined by acid hydrolysis is likely to be the combination of non-starch and starch lipids.



Abbreviation used for lipid standards

TAG	Triacylglycerol	PE	Phosphatidyl-ethanolamine
DAG	Diacylglycerol	PC	phosphatidylcholine
MAG	Monoacylglycerol	PI	Phosphatidylinositol
MGDG	Monogalactosyl diglyceride	LPE	Lysophosphatidyl-ethanolamine
DGDG	Digalactosyl diglyceride	LPC	Lysophosphatidyl-choline

Figure 8.2 Standard curves and correlation coefficients for lipids used as standards in the HPLC analysis of lipid components

The ratios of total lipid from the petroleum ether, chloroform and ethanol extraction to the acid hydrolysis are 46.3, 72.5 and 80.6% respectively, which are represented to the proportion of non-starch lipid in the total lipid. Comparing to the proportions of non-starch lipid in the total of starch and non-starch lipid (63.1–70.5%) reported by Morrison et al. (1975), petroleum ether is not effective in extracting all lipids from flour whilst chloroform can extract most of non-starch lipid in flour that has been reported previously and a small amount of starch lipid in flour could be extracted with ethanol at room temperature.

Table 8.1 Composition of flour lipids from a single solvent extraction

	Petroleum ether	Chloroform	Ethanol (RT)	AACC 30-10
Relative polarity*	0.117	0.259	0.654	-
Total lipid (%, w/w)	0.74	1.16	1.29	1.60
Lipid classes in lipid extract (%, w/w of total lipid)				
Non-polar lipids	96.4	94.0	90.1	-
Glycolipids	3.3	5.8	9.1	-
Phospholipids	0.3	0.2	0.7	-

Note: * Source: (Reichardt, 1988)
Non-polar lipids are calculated as a sum of TAG, DAG and MAG
Glycolipids are calculated as a sum of MGDG and DGDG
Phospholipids are calculated as a sum of PE, PI, PS, PC, LPE and LPC
Abbreviation RT refers to room temperature

Lipid extraction for flour was dependant on the polarity of the solvent, with higher polarity resulting in better extraction (Table 8.1). A similar effect of solvent polarity on the lipid extraction was found for Swedish winter wheat flour (Georgopoulos et al., 2006) although the lipid content of flour in the current study was higher. It is also possible that the observations could be due to lipid levels varying between wheat varieties. The amount of lipid extracted using petroleum ether (low polarity) was two thirds of that extracted using ethanol (high polarity). The level of lipids extracted using chloroform is about 90% of that extracted using ethanol. Most of the lipid extracted by petroleum ether is free lipid and ethanol can extract both free and bound lipids (Cornell and Hoveling, 1998b; Chung and Ohm, 2000). The result of high levels of lipid

extracted using ethanol was expected, as this solvent is able to extract a higher proportion of bound lipid.

The composition of lipids extracted from the flour was dependant on the polarity of solvent, with the higher polarity extracting a higher proportion of glycolipids and phospholipids (Table 8.1). Of the three solvents, the highest level of polar lipids (glycolipids and phospholipids) was extracted using ethanol (9.8%), following by chloroform (6.0%) and petroleum ether (3.6%). These results confirm the trend found in the lipid extraction of Swedish flours reported by Georgopoulos et al. (2006). The proportion of non-polar lipid extracted in the current study (90–96%) was higher than that found by McCormack et al. (1991) (60–74%).

8.4 Sequential solvent extraction of lipid in flour

The differential selectivity of solvents can be utilized to extract free and bound lipids from flour. The sequential solvent extraction of lipid in flour was performed firstly with petroleum ether, followed by chloroform and finally with ethanol at room temperature. The purpose of developing a sequential solvent extraction was: 1) to remove abundant non-polar lipid that affects the sensitivity of detection of glycolipids and phospholipids; 2) to enrich the glycolipids and phospholipids present at low levels in flour and 3) to separately extract free and bound lipids from flour.

8.4.1 Optimisation of the sequential solvent extraction condition

In order to investigate and optimise the conditions for sequential extraction, lipid was extracted using flour S008 and the procedure of single solvent extraction described in section 7.5.2. Four variations of solvent extraction steps were applied (Table 8.2). The results were assessed on the basis of the total extractable lipid and the level lipid successfully extracted at each extraction step. The total extracted from sequential solvent extraction (1.27–1.46%) was higher than from the single solvent extraction using chloroform and ethanol (1.16–1.29%) and closer to the value of total lipid determined by the AACC method (1.6%) (Tables 8.1 and 8.2).

Table 8.2 Varying solvent steps in the sequential solvent extraction method

	Solvent steps	Lipid extract (%, w/w)
Test 1	Petroleum ether x 1 extraction	0.43
	Chloroform x 1 extraction	0.41
	Ethanol x 1 extraction	0.49
	Total extractable lipid	**1.33**
Test 2	Petroleum ether x 2 extractions	0.74
	Ethanol 1st x1 extraction	0.39
	Ethanol 2nd x 1 extraction	0.14
	Total extractable lipid	**1.27**
Test 3	Petroleum ether x 2 extractions	0.73
	Chloroform x 1 extraction	0.19
	Ethanol x 1 extraction	0.46
	Total extractable lipid	**1.37**
Test 4	Petroleum ether x 3 extractions	0.79
	Chloroform x 1 extraction	0.17
	Ethanol x 1 extraction	0.50
	Total extractable lipid	**1.46**

In the context of sequential extraction, a single treatment using petroleum ether was insufficient to remove all of the petroleum ether extractable lipid from flour. Following the single treatment, the remaining lipid interfered with the subsequent extraction at the next step, resulting in a high level of lipid extracted during the chloroform step (test 1). A two-time extraction using petroleum ether was able to extract most of the petroleum ether extractable lipid from flour, as the level of lipid extracted at the chloroform step (g/100 g of flour) reduced from 0.41 (test 1) to 0.19 (test 3). A three-time extraction using petroleum ether did not extract any further lipid, as the levels of lipid extracted at the petroleum ether and chloroform steps in test 3 and 4 were similar. In addition, the use of ethanol immediately after petroleum ether did not improve the extraction efficiency. The sequential solvent extraction (test 3) using 2 x petroleum ether, 1 x

chloroform and 1 x ethanol was effective at removing all of the extractable lipid material from the flour.

8.4.2 Lipid classes in the sequential solvent extraction of lipid in flour

Following the establishment of a procedure for sequential extraction using a commercial flour, further studies utilised flours of two particular cultivars. These were chosen because they are from two main types of wheat: soft wheat (Rosella cv) and hard wheat (Lang). Furthermore, flour was specially milled in a single variety to avoid the effect of various compositions of protein and lipid from different wheat varieties on the interaction study. Lipids in flour (cv Lang) were extracted using the solvent steps described as test 3 in the procedure described in Table 8.2. The individual components of the lipid classes in the three lipid extracts of the sequential solvent extraction were determined using HPLC and TLC. The chromatogram and TLC results of the three lipid extracts are illustrated in Figure 8.1.

A large amount of non-polar lipid and small quantities of glycolipids were extracted during the petroleum ether step, glycolipids and some phospholipids were extracted with chloroform and phospholipids were the main component obtained with ethanol. These results were also confirmed by lipid analysis on TLC. The petroleum ether extract contained a high proportion of TAG and DAG (Fig 8.3, lane a), MGDG and DGDG were the major constituents of the chloroform extract (lane b) and LPC dominated in the ethanol extract (lane c). These results are consistent with the findings from the single solvent extraction (Section 8.1), indicating the high extractability of polar lipids using chloroform and ethanol. The removal of non-polar lipid using petroleum ether resulted in the enrichment of glycolipids and phospholipids in the chloroform and ethanol extraction and thus they were readily detected by HPLC. The composition of free lipid in this study was similar to those reported by Ohm and Chung (2002) and Konopka et al. (2006). The sequential solvent extraction appeared to be effective in extracting free and bound lipid from flour for determining lipid classes on HPLC.

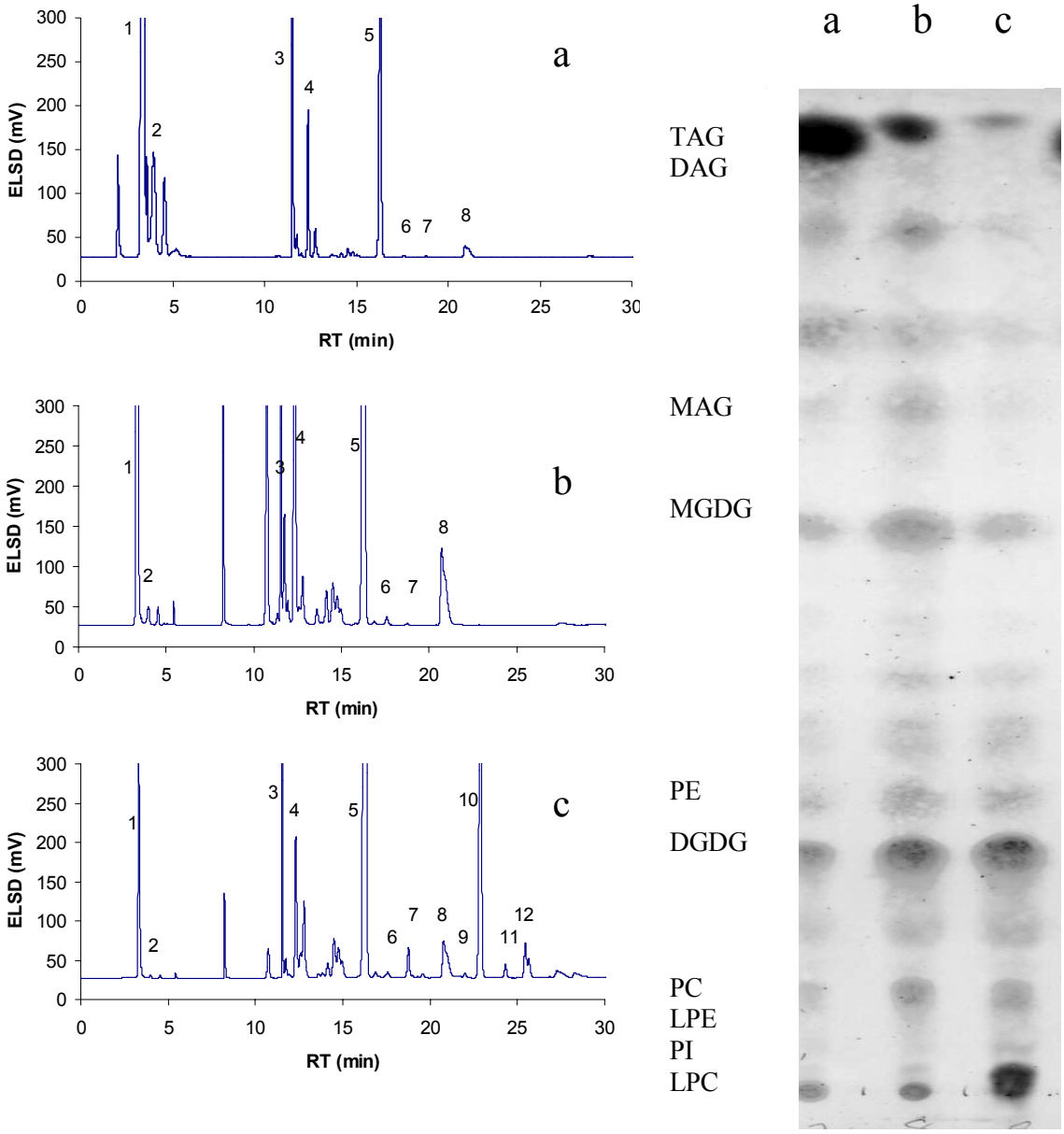


Figure 8.3 Identification of lipid classes in flour (cv Lang) extracted with the three-step solvent extraction using HPLC and TLC

Notes
1 Triacylglycerol (TAG)
2 Diacylglycerol (DAG)
3 Monoacylglycerol (MAG) and solvent
4 Monogalactosyl diglyceride (MGDG)
5 Digalactosyl diglyceride (DGDG)
6 Phosphatidylethanolamine (PE)
7 Phosphatidylinositol (PI)
8 Phosphatidylserine (PS) and phosphatidylcholine (PC)
9 Lysophosphatidylethanolamine (LPE)
10 Unknown, possibly Lysophosphatidylcholine (LPC)
11 Unknown
12 Unknown
a Lipid extract from the petroleum ether step
b Lipid extract from the chloroform step
c Lipid extract from the ethanol step (room temperature)

8.5 A selective solvent extraction of lipid in gluten

Gluten was prepared on a laboratory scale from the flour of a single variety (cv. Lang) using the procedure described in Section 7.3. The validated extraction conditions used earlier for the sequential solvent extraction of flour samples (test 3) was used to assess the applicability of the procedure to gluten samples. Lipid classes in the extracts were analysed using the HPLC and TLC methods described in Section 7.5. The total lipid content of gluten was determined with the AACC acid hydrolysis method. This value is used for comparative purposes in the validation of the extraction method.

8.5.1 Effect of temperature on the ethanol extraction step

Total amount of lipid extracted from gluten using the sequential solvent extraction at room temperature (4.18%) was substantially lower than the total lipid determined by the acid hydrolysis method (6.30%) (Table 8.3). This indicates that some lipids might not be accessible with polar solvents including ethanol at room temperature. It has previously been reported that, at higher temperatures (90–100°C), ethanol is able to extract lipid from within starch granules in cereal products (Morrison and Coventry, 1985), so it was decided to compare the use of ethanol at two temperatures to extract lipid from gluten. Hence, the selective solvent extraction was modified with the use of hot ethanol (70°C) at the ethanol step extraction. This resulted in extraction of higher amounts of lipid (4.36%) from gluten compared to the room temperature ethanol step (1.32%) (Table 8.3). As a result, total lipid extracted using the modified three-step extraction (7.02%) was slightly higher than the amount of lipid analysed by acid hydrolysis (6.30%) and in the range of total lipid values of gluten (5–10%) reported by Dill (1925); Olcott and Mecham (1947) and Ponte et al. (1967).

Table 8.3 Effect of ethanol temperature in the selective solvent extraction of lipids from gluten

Extraction procedure	Lipid content (%, w/w)		
	Three-step solvent extraction		Acid hydrolysis
	RT ethanol	Hot ethanol	
Total	4.18	7.02	6.30
In each successive solvent			
Petroleum ether	1.70	1.93	
Chloroform	1.15	0.73	
Ethanol	1.32	4.36	

Note Abbreviation RT refers to room temperature

8.5.2 Lipid composition of the selective solvent extracts from gluten

When the various lipid extracts were analysed by HPLC (Figure 8.4) only non-polar lipids were extracted during the petroleum ether step whereas chloroform and ethanol (70°C) could extract non-polar lipid as well as glycolipids and phospholipids. The lipid classes obtained from the chloroform and ethanol extraction steps were very similar. They consisted of predominantly non-polar lipid and glycolipids and a trace of phospholipids, and this was confirmed by the TLC analysis (Figure 8.4). Although the chloroform and ethanol extracted a similar pattern of lipids, hot ethanol was far more effective, as it was shown to extract 4.36% compared to 0.73% for chloroform (Table 8.3). The non-polar lipid extracted at the petroleum ether step is most likely to be free lipid present in gluten, as it can be extracted readily with a solvent of low polarity. On the other hand, the non-polar components in the lipid extracted with chloroform and ethanol might be present as bound lipids in gluten as they were only extracted with polar solvents.

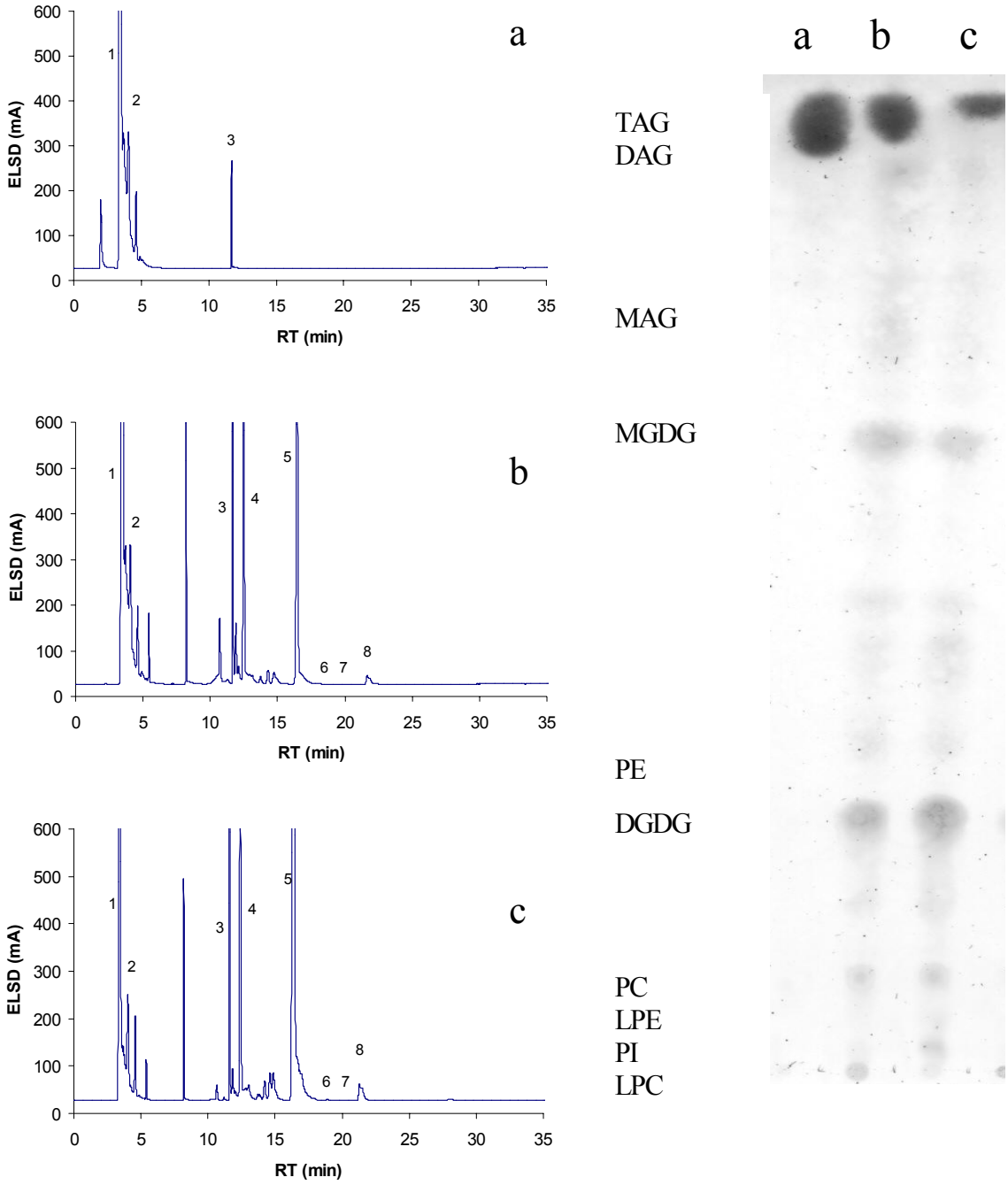


Figure 8.4 The identification of lipid components using HPLC and TLC for gluten (cv Lang) following selective solvent extraction

Notes
1 Triacylglycerol (TAG)
2 Diacylglycerol (DAG)
3 Monoacylglycerol (MAG) and solvent
4 Monogalactosyl diglyceride (MGDG)
5 Digalactosyl diglyceride (DGDG)
6 Phosphatidylethanolamine (PE)
7 Phosphatidylinositol (PI)
8 Phosphatidylserine (PS) and phosphatidylcholine (PC)
a Lipid extract from the petroleum ether step
b Lipid extract from the chloroform step
c Lipid extract from hot ethanol extraction

The ethanol extract from flour contained three unidentified peaks (Figure 8.3, peak 10, 11 and 12), however, these were absent for all extracts derived from gluten. The absence of these peaks in gluten lipid could be related to the removal of starch during gluten preparation. The fact that these lipids appeared as peaks with relatively long retention times suggests that they are polar lipids, most likely phospholipids, which could be washed out during isolation of gluten from flour as they are associated with starch. Lysophosphatidylcholine is a major starch lipid in flour that can be extracted with hot ethanol (Morrison et al., 1975). As peak 10 was a dominant peak in the ethanol extract of flour but not present in the ethanol extract of gluten (Figure 8.3), it is most likely to be lysophosphatidylcholine.

These results indicate that the sequential solvent extraction can be used to separately extract free and bound lipid in flour and gluten based on the extractability with solvents of different polarity. The lipid extracted using petroleum ether is free lipid, while the sum of lipid extracted using chloroform and ethanol is bound lipid.

8.6 Lipid composition in flour and gluten of hard and soft wheat

The procedures described here have been validated for the measurement of the amount of free and bound lipid, as well as their individual components, in both flour and gluten, and so these are now applied to compare flour and gluten from hard (cv. Lang) and soft wheat (cv. Rosella) varieties. The lipid composition, moisture content and protein content of flour and gluten from hard and soft wheat are presented in Table 8.4. The total lipid values of flours found in the current study (1.39–1.56%) are in the range of total lipid reported previously (1.4–2.0%) (Chung, 1986). Although there are similar amounts of glycolipids and phospholipids in the free and bound forms, flours of this study have a higher proportion of free non-polar lipids and those of bound non-polar lipid are lower compared to the reported values by Chung (1986), in which the proportion of free and bound non-polar lipid in flour ranged from 0.5–0.7% and 0.1–0.3%, respectively.

The composition of free and bound lipids in flour and gluten are compared within and between the two wheat cultivars (Table 8.4). As expected, total extractable lipid of flour was higher in soft wheat variety than in hard wheat variety. Accordingly gluten

prepared from soft wheat flour contained higher lipid content than that from hard wheat flour. Although there are variations in the lipid content, both soft and hard wheat flour contained a higher proportion of free lipid (64–66%) compared to gluten (28–30%). The ratio of free to bound lipid in soft and hard wheat flour was approximately 2:1 whilst in gluten of both types of wheat was 1:2, that is consistent with reports by Olcott and Mecham (1947) and Chung (1986). A reduction in the free lipid content of gluten, along with an increase of protein content has been shown to be involved in the association of lipids with gluten proteins.

Table 8.4 Lipid composition and values of some components in flour and gluten

	Hard wheat (cv Lang)		**Soft wheat (cv Rosella)**	
	Flour	Gluten	Flour	Gluten
Yield of gluten (%, w/w)	15.4	-	12.0	-
Moisture content (%, w/w)	14.0	7.0	13.5	7.9
Protein content (%, w/w)	12.8	70.3	10.8	73.9
Total extractable lipid (%, w/w)	1.39	7.03	1.56	8.48
In each successive solvent (%, w/w of total lipid)				
Petroleum ether (%)	64	28	66	30
Chloroform (%)	16	10	15	12
Ethanol (%)	20	62	19	58
Free lipid (%, w/w)	**0.89**	**1.93**	**1.03**	**2.59**
*Non-polar lipid**	*(85)*	*(100)*	*(79)*	*(100)*
*Glycolipid**	*(12)*	*(0)*	*(18)*	*(0)*
*Phospholipid**	*(2)*	*(0)*	*(3)*	*(0)*
Bound lipid (%, w/w)	**0.51**	**5.09**	**0.53**	**5.89**
*Non-polar lipid**	*(14)*	*(71)*	*(13)*	*(65)*
*Glycolipid**	*(35)*	*(23)*	*(40)*	*(29)*
*Phospholipid**	*(51)*	*(6)*	*(47)*	*(6)*

Note: (*) The proportion of lipid classes in free or bound lipids expressed as a percentage

Although the soft wheat contained higher total lipid levels in flour and gluten compared to the hard wheat, the lipid distribution in flour or gluten were similar for hard and soft

wheat (Table 8.4). The proportion of non-polar lipids, glycolipids and phospholipids in the free and bound lipid extract were similar in flour and gluten of both soft and hard wheat varieties. The results suggest that while there may be differences in total lipids in flour between wheat varieties, the composition of lipids in flour and gluten remains similar.

The distribution of lipid classes in free and bound lipids was different between flour and gluten. For the hard wheat variety, free lipid of flour contained predominantly non-polar lipids (85%) and small amounts of glycolipids (12%) and phospholipids (2%) while only non-polar lipid (100%) existed as a free lipid in gluten (Table 8.4). Bound lipid of flour and gluten contained all of lipid classes but in differing proportions. The highest proportion of bound lipid in flour was phospholipids (51%) whilst it was non-polar lipid in gluten (71%). Again, the distribution of lipid classes in free and bound lipids was similar in the soft and hard wheat varieties.

8.7 Distribution of lipid classes in flour and gluten in relation to protein and lipid interactions

It might be expected that when gluten is isolated from flour, the distribution of free and bound lipids would alter. In order to understand the changes in this distribution, the quantities of total, free and bound lipids were calculated for 100 g of flour as well as for the gluten. It is emphasised that these data have been presented for the amount of gluten obtained from 100 g of flour, to facilitate direct comparison (Tables 8.5 and 8.6). The lipid content remaining in gluten was approximately 65–75% of that in the original flour for both varieties. Virtually all of the non-polar lipids initially in flour have been retained in the gluten. Of these lipids, approximately 50–60 % of free form found in flour became bound with gluten components. This appears to confirm the previous report that during dough mixing, approximately a half of the free lipid in flour became bound and could not be extracted with ether (Olcott and Mecham, 1947). In the current study, the differences in lipid content primarily involved glycolipids and phospholipids, and as a result, the gluten contained a higher proportion of non-polar lipids. Approximately half of the glycolipids and only a small amount of phospholipids from flour were found in gluten (Tables 8.5 and 8.6).

Comparison of the protein pattern of flour and gluten on SDS-PAGE showed very similar results (Figure 8.5). The only differences between gluten and flour were noted in the albumin and globulin region. As albumins and globulins are water-soluble proteins, it might be expected that these would be removed from gluten during the gluten washing process.

Although the flour and gluten contained similar protein profiles, the distribution of free and bound non-polar lipids in flour and gluten was different. Of the non-polar lipid present in flour, 92% existed as free lipid compared to 35% in gluten. Conversely, the flour contained low levels of bound non-polar lipid (8%), compared to gluten (65%). The results indicate that a large amount of non-polar lipids in flour became associated with the proteins during gluten preparation.

Table 8.5 Distribution of lipid classes in flour and the corresponding gluten for cv Lang

	Flour *(100 g of flour)*		**Gluten** *(from 100 g of flour)*	
Total lipid	**1.39**	**(100)**	**1.05**	**(100)**
Total non-polar lipids	0.83	(60)	0.83	(79)
Total glycolipids	0.29	(21)	0.18	(17)
Total phospholipids	0.28	(19)	0.05	(5)
Total non-polar lipids	**0.83**	**(100)**	**0.83**	**(100)**
Free-form	0.76	(92)	0.29	(35)
Bound-form	0.07	(8)	0.54	(65)
Total glycolipids	**0.29**	**(100)**	**0.18**	**(100)**
Free-form	0.11	(38)	0.00	(0)
Bound-form	0.18	(62)	0.18	(100)
Total phospholipids	**0.28**	**(100)**	**0.05**	**(100)**
Free-form	0.02	(7)	0.00	(0)
Bound-form	0.26	(93)	0.05	(100)

Notes: Values are presented in units of g and results in parentheses are the proportion expressed as a percentage of the total

All of the glycolipids and phospholipids present in gluten were in the bound form. For both the soft and hard wheat varieties, the amount of bound glycolipids retained in gluten was the same as the original amount of bound glycolipids in flour whilst the amount of bound phospholipids was very much lower in gluten than in flour (Table 8.5 and 8.6). It has been reported that the majority of glycolipids in flour are non-starch lipid and very small amount is present in starch lipids while phospholipids have been found predominate in starch lipids (Morrison et al., 1975). The low level of phospholipids in gluten could be due to those lipids being associated with starch granules (Morrison, 1988) that may be removed during gluten preparation. Of the total glycolipid, approximately 40% is present in the free form and 60% as the bound form, which is consistent with previous findings (Chung, 1986). If all free glycolipids are removed with starch and other components during the gluten isolation, the presence of the same amount of bound glycolipids in flour and gluten suggests that there is no further interaction of free glycolipids with gluten.

Table 8.6 Distribution of lipid classes in flour and the corresponding gluten for cv Rosella

	Flour *(100 g of flour)*		**Gluten** *(from 100 g of flour)*	
Total lipid	**1.56**	**(100)**	**1.02**	**(100)**
Total non-polar lipids	0.88	(76)	0.77	(75)
Total glycolipids	0.40	(20)	0.20	(20)
Total phospholipids	0.28	(4)	0.04	(5)
Total non-polar lipids	**0.88**	**(100)**	**0.77**	**(100)**
Free-form	0.81	(92)	0.31	(40)
Bound-form	0.07	(8)	0.46	(60)
Total glycolipids	**0.40**	**(100)**	**0.20**	**(100)**
Free-form	0.19	(48)	0.00	(0)
Bound-form	0.21	(52)	0.20	(100)
Total phospholipids	**0.28**	**(100)**	**0.04**	**(100)**
Free-form	0.03	(11)	0.00	(0)
Bound-form	0.25	(89)	0.04	(100)

Notes: Values are presented in units of g and results in parentheses are the proportion expressed as a percentage of the total

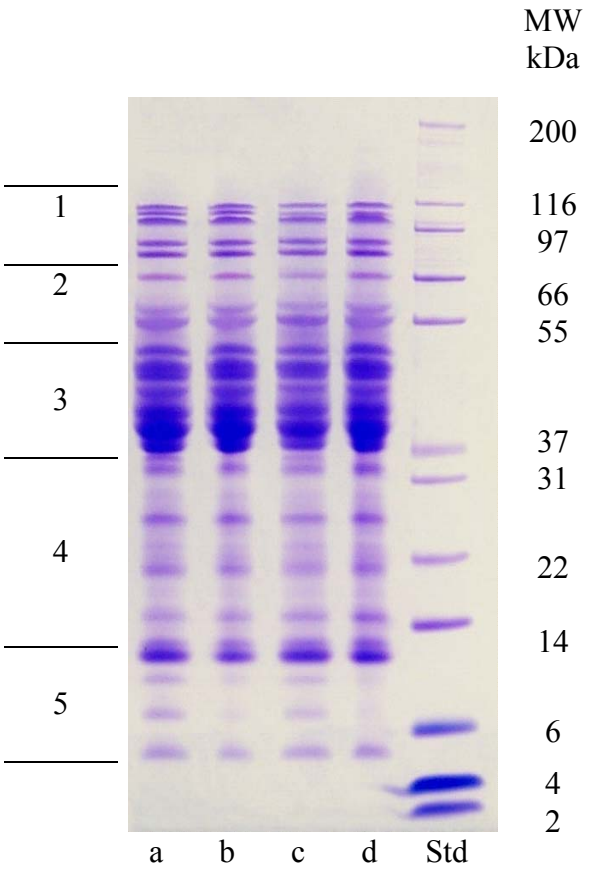


Figure 8.5 SDS-PAGE pattern of protein in flour and gluten samples

Notes				
	a	Flour (cv Lang)	b	Gluten (cv. Lang)
	c	Flour (cv Rosella)	d	Gluten (cv Rosella)
	Std	Protein standard	MW	Molecular weight
	1	Region of HMW-GS	2	Region of ω-gliadin
	3 and 4	Region of α,β,γ-gliadin and LMW-GS	5	Region of albumin and globulin

8.8 Summary of results for extractability and composition of lipids in flour and gluten

The solvent extraction method, based on the three-step treatment with petroleum ether, chloroform and ethanol, was effective in separately extracting free and bound lipid components from flour. Whereas for flour, extraction was achieved at room temperature, it was shown that a temperature of 70°C was required at the ethanol step for efficient extraction of the bound lipid from gluten. The selective solvent extraction of gluten showed that chloroform extracted only small amounts of lipid and this was composed of similar classes of lipid to those subsequently extracted with ethanol. Therefore, this indicates that the chloroform step might be eliminated for the lipid extraction of gluten and gluten fractions in later phases of this investigation.

Comparing the composition of free and bound lipid from flour and gluten indicates that the proportion of bound lipids was higher in gluten than in flour. Most of the non-polar lipids originally found in the flour were retained in the gluten with an increasing proportion of bound form. A small proportion of the glycolipids and phospholipids from flour were recovered in the gluten but all existed in the bound form. Two alternative explanations of these observations might be proposed: non-polar lipids could be entrapped within the gluten structure or they may become closely associated with at least some of the protein components of gluten. On the basis of the relatively small amounts of glycolipids and phospholipids remaining in gluten following the gluten preparation, there is a lack of evidence that the free form of these lipids further interact with gluten components. In addition, there were no obvious differences in the distribution of protein and lipid components between the hard and soft wheat samples. On this basis, along with the higher protein content and also yield of gluten of the hard wheat flour, this was chosen for the further study of protein-lipid interactions in gluten.

Chapter 9

Results and discussion: Effect of acetic acid concentration on protein solubility and lipid distribution in gluten fractions

9.1 Introduction

The study of extractability and composition of lipid in flour and gluten (Chapter 8) demonstrated that approximately 65–75% of flour lipid remained in the gluten and existed primarily in the bound form. Most of the non-polar lipid originally found in flour as free lipid was retained in gluten as bound lipid. Only half of the glycolipid and a small amount of phospholipid in flour remained in the gluten and all occurred in the bound form. It was noted that the amount of bound glycolipids in gluten was similar to that present in flour. These results indicated that associations developed between the lipid and protein during gluten preparation.

Physical techniques including phosphorus nuclear magnetic resonance spectroscopy and freeze-fracture electron microscopy (Marion et al., 1987) as well as electron spin resonance (Hargreaves et al., 1994) have been applied to the investigation of the interaction of protein and lipid in gluten. However the most widely used approach has involved protein fractionation (Olcott and Mecham, 1947; Ponte et al., 1967; Hoseney et al., 1970a). The association of lipid with glutenin or gliadin has been found to depend on the particular protein fractionation method used (Chung, 1986; Carr et al., 1992). It has been demonstrated that both polar and hydrophobic bonds participate in the interactions of lipid and protein (Wehrli and Pomeranz, 1970). On the other hand, the physical studies indicated that the interaction of protein and lipid in the gluten network involved the physical entrapment of lipid and also polar or ionic bonds between protein and lipid components (Carr et al., 1992). At this time, the mechanism (or mechanisms) contributing to the protein-lipid interaction in gluten has not been fully elucidated.

For many decades, it has been known that although gluten is a water-insoluble substance, it can be partially dissolved in a dilute acetic acid solution. This solubilisation could be due to the effect of the acidic conditions on changing structure

and net charge of the protein molecules (Damodaran, 1996) as well as the disruption of hydrogen bonds within the gluten matrix (Wrigley et al., 2006). Various acetic acid concentrations have been used to selectively separate gliadin and glutenin fractions (Berot et al., 1994). These effects of acetic acid on the structure and composition of gluten protein could have an impact on the distribution of free and bound lipid, which might be related to the interaction of protein and lipid in gluten.

In this study, experiments were carried out to investigate the effect of acetic acid on the distribution of lipid and protein in gluten samples prepared with two different methods. Petroleum ether defatted gluten was used as gluten control and as material for all treatments in this study. The first method was to treat gluten with acetic acid at two different concentrations (procedure in Section 7.3.3). The second method was to fractionate gluten into supernatant and pellet using a single acetic acid fractionation method at various concentrations of acetic acid (procedure in Section 7.3.4). The changes of lipid and protein distribution under acidic treatment and fractionation will be discussed in relation to the interactions of protein and lipid.

9.2 The alteration of protein and lipid composition in gluten with acetic acid treatments

9.2.1 Protein composition of acetic acid treated gluten

Gluten samples were separately treated with 0.01 and 0.1 M acetic acid and then freeze-dried. Protein and moisture contents of the acetic acid treated gluten were determined and the parameters of these treatments are presented in Table 9.1. After treatment with 0.01 and 0.1 M acetic acid, yields of 89.9 and 92.1% were obtained, respectively. The protein and moisture contents of these gluten samples were slightly higher than that of the gluten control. The higher moisture content of acetic acid treated gluten could be due to a residue of acid in these gluten samples that was removed with water during the moisture determination. As expected, protein recovery values were high at 95.6 and 96.7% for 0.01 and 0.1 M acetic acid treated gluten samples, respectively.

The protein profile of gluten control and acetic acid treated glutens was investigated using electrophoresis under reduced and non-reduced conditions and these SDS-PAGE gels are displayed in Figure 9.1. Under reducing conditions, dithiothreitol was used as

reducing agent in order to break the disulfide bonds within and between subunits of glutenins. Therefore, a number of protein bands can be visualised on the reduced SDS-PAGE gel, which ranged from 116 to 12 kDa and included HMW-GS in region 1, ω-gliadins in region 2 and α,β,γ-gliadin and LMW-GS in regions 3 and 4.

Table 9.1 Protein content in gluten control and acetic acid treated gluten

	Gluten control	0.01 M acetic acid treated gluten	0.1 M acetic acid treated gluten
Moisture content (%, w/w)	8.32	11.3	11.1
Yield (%, w/w)	-	89.8	92.1
Protein content (%, w/w)	70.6	75.2	74.2
Protein recovery (%, w/w)	-	95.6	96.7

Under non-reducing conditions, several distinct protein bands were observed in the range of MW between 22–66 kDa. These bands possibly belong to the gliadin type of protein because gliadins are known to be monomeric proteins. They consist of α,β,γ-gliadins which are only associated through the intra-molecular disulfide bonds and ω-gliadins that are not able to form disulfide bonds due to a lack of cysteine residues (Shewry and Tatham, 1997; Wrigley et al., 1998). On another hand, glutenins occur in the polymeric form, in which the HMW-GS and LMW-GS are associated through the inter-molecular and extra-molecular disulfide bonds (Shewry et al., 1992). Therefore, these proteins could not enter the gel and their protein bands do not appear on the non-reduced gel.

In a comparison of the protein pattern between gluten control and acetic acid treated glutens, no differences were found between the reduced and non-reduced SDS PAGE gels (Figure 9.1). The results from SE-HPLC chromatography provided similar observations, with a similar peak pattern presented in the gluten control and acetic acid treated glutens (Figure 9.2). This consisted of the first peak corresponding to polymeric protein and the second peak representing monomeric protein. The ratios of monomeric to polymeric protein of these gluten samples were similar with values of approximately 1.8. These results were expected because acetic acid is a weak acid and it can alter the

folding structure of proteins, thereby affecting the solubility of proteins (Damodaran, 1996). Under condition of the current treatment, acetic acid cannot cause the hydrolysis of protein, hence the protein pattern of acetic acid treated gluten was expected to be similar to that of gluten control.

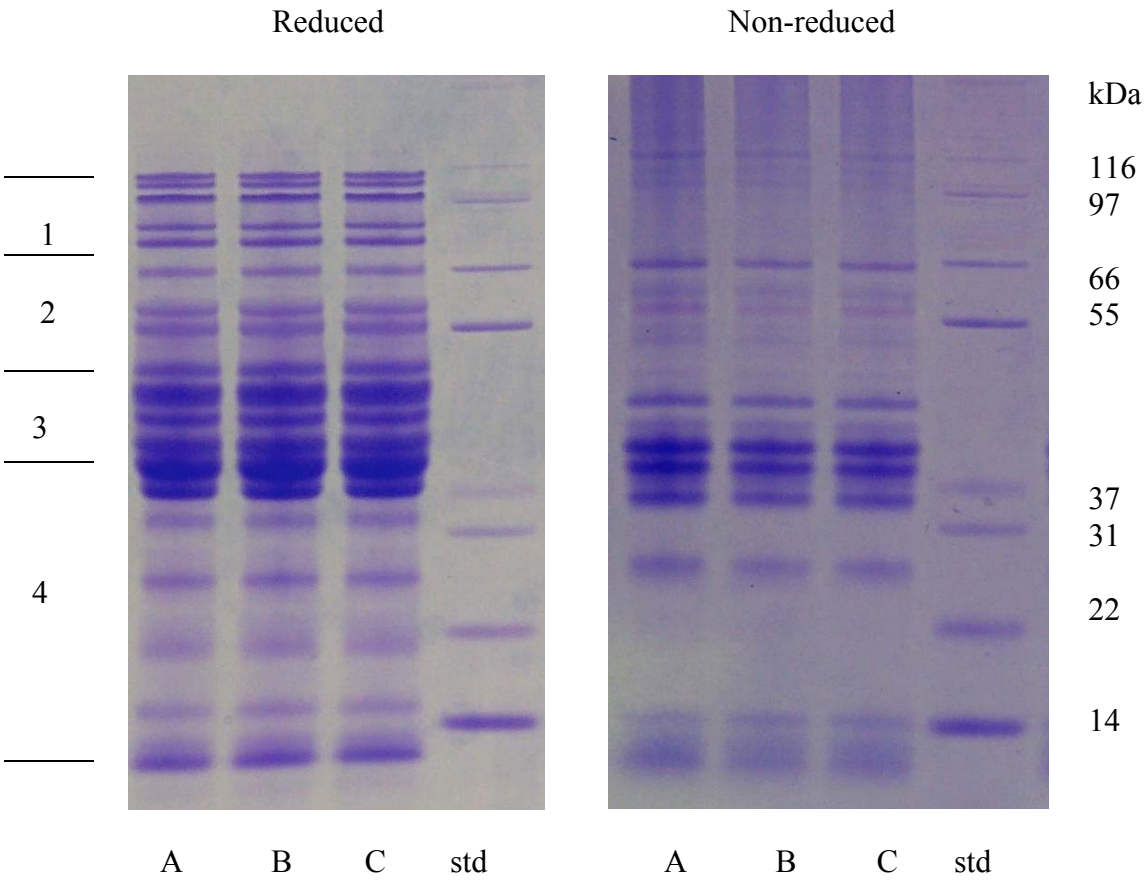


Figure 9.1 Protein pattern of gluten samples on SDS-PAGE

Notes	A	0.01 M acetic acid treated gluten
	B	0.1 M acetic acid treated gluten
	C	Gluten control
	Std	Protein standard
	1	Region of HMW-GS
	2	Region of ω-gliadin
	3 &4	Region of α,β,γ-gliadin and LMW-GS

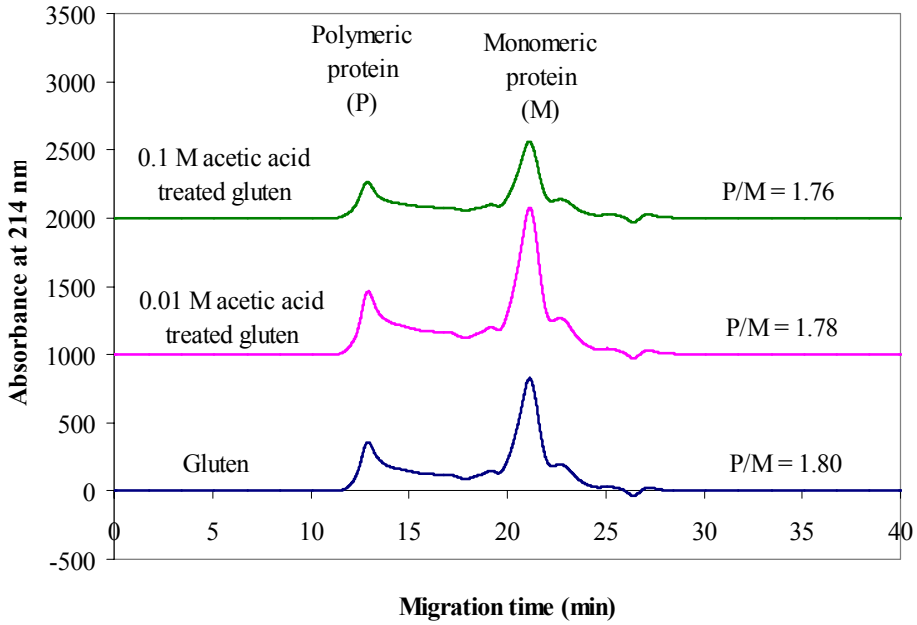


Figure 9.2 SE-HPLC protein profile of acetic acid treated glutens

9.2.2 Lipid composition of acetic acid treated gluten

Free and bound lipids were extracted from the gluten control and the acetic acid treated gluten samples. The lipid contents of these samples are presented in Table 9.2. The total extractable lipid in gluten treated with both acetic acid concentrations was similar (4.21 and 4.58%) but lower than that in gluten control (5.70%). After treating gluten with acetic acid, even at short periods of contact, lipid became more readily extractable with solvents of low polarity. This resulted in an increase of amount of free lipid in the acetic acid treated gluten (1.25–1.44%) compared to that in the gluten control (0.25%) (Table 9.2). This could be due to the effect of acetic acid on changing the structure of gluten, therefore, the bound lipid originally found in gluten became accessible to solvent during lipid extraction.

It is noted that in the current study the time of mixing was carefully controlled at two minutes. Previously it was reported that contact between gluten and diluted hydrochloric acid for longer periods, of up to forty eight hours, could result in a deterioration in functional properties of the gluten (MacRitchie, 1985). Therefore, the

periods of mixing gluten with acid solution might have an important role in changing the gluten structure and also in the distribution of free and bound lipid. Further study on the influence of various mixing times might provide additional evidence on the effect of acetic acid on gluten structure and lipid distribution.

Table 9.2 Free and bound lipids in gluten control and acetic acid treated gluten

	Gluten control	0.01 M acetic acid treated gluten	0.1 M acetic acid treated gluten
Total lipid (%, w/w)	**5.70**	**4.21**	**4.58**
Free lipid (%, w/w)	**0.23**	**1.11**	**1.27**
Non-polar lipids	(100)	(100)	(92)
Glycolipids	(0)	(0)	(8)
Phospholipids	(0)	(0)	(0)
Bound lipid (%, w/w)	**5.47**	**3.10**	**3.31**
Non-polar lipids	(50)	(39)	(34)
Glycolipids	(38)	(53)	(56)
Phospholipids	(12)	(8)	(11)

Notes: Total lipid is calculated based on the amounts of free lipid and bound lipid.
Non-polar lipids are TAG, DAG and MAG.
Glycolipids are MGDG and DGDG.
Phospholipids are PE, PI, PS, PC, LPE and LPC
Data in parentheses represent proportions expressed as a percentage of the lipid

Lipid classes of free and bound lipids in the gluten control and acetic acid treated gluten were analysed and composition of these lipids are presented in Table 9.2. Of the free lipids, non-polar lipids were the main component in all gluten samples although very small amounts of glycolipid were found in the 0.1 M acetic acid treated gluten. In addition, all lipid classes were found in the bound lipid extract of all the gluten samples but at varying proportions. Of the total bound lipid, the gluten control had a high proportion of non-polar lipids whilst acetic acid treated gluten contained large proportion of glycolipids (Table 9.2). This could be a consequence of an increase of free non-polar lipid level in acetic acid treated gluten.

A change in the distribution of the lipid classes was also observed through the proportion of lipid components in the total lipid (Figure 9.3). This alteration primarily occurred in non-polar lipid after gluten was treated with acetic acid, demonstrating that the level of free non-polar lipid increased and consequently the amount of bound lipid declined after acetic acid treatments. Non-polar lipids have been found to be associated with gluten components during gluten preparation (Chapter 8). These associations could be formed during the hydration of flour, when a number of hydrogen bonds have been formed within and between gliadins and glutenins. Non-polar lipids are substances of low polarity, therefore under aqueous conditions they tend to associate with gluten components of low polarity through hydrophobic interactions. During gluten preparation some of these lipids might either be entrapped in the gluten matrix or associate with gluten components. Under treatment with acetic acid, some hydrogen bonds can be disrupted and the gluten structure is re-orientated (Wrigley et al., 2006). As a result, these non-polar lipids that have been held within the gluten matrix became accessible to solvent molecules and were thereby extracted as free lipid.

Acetic acid treatments did not show any effect on the distribution of glycolipids and phospholipids, demonstrated by the similar proportion of these lipids in free and bound forms of all gluten samples (Figure 9.3). It has been found that the amounts of bound glycolipids remaining in gluten were the same as those originally present in flour (Chapter 8). These results indicate that glycolipids and phospholipids might associate with gluten protein in specific ways that were not affected under acidic conditions. It was also noted that levels of free and bound lipid (Table 9.2) and the distribution of lipid classes (Figure 9.3) were similar for both 0.01 and 0.1 M acetic acid treated glutens. This indicates that both acetic acid concentrations have the same effect on changing the extractability of gluten lipid.

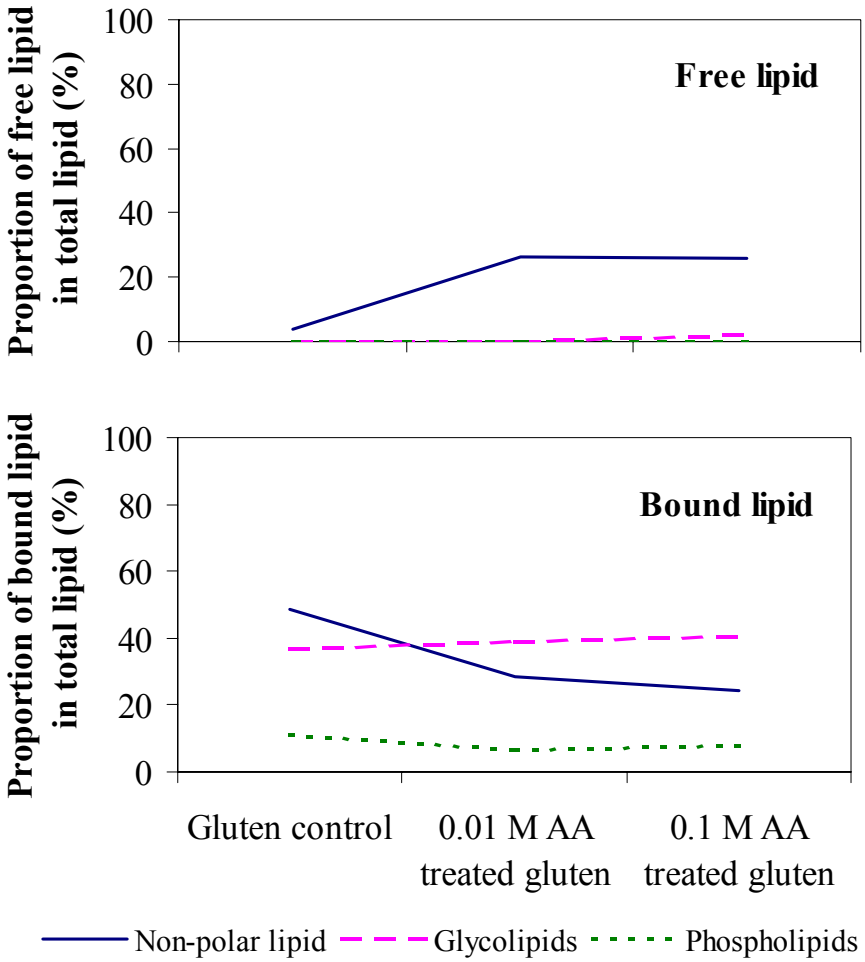


Figure 9.3 Distribution of lipid classes in gluten control and acetic acid treated gluten
Note AA represents acetic acid

9.3 Protein and lipid distribution in acetic acid gluten fractions

9.3.1 Effect of acetic acid concentration on protein distribution

Each gluten sample was fractionated into supernatant and pellet with three different concentrations of acetic acid (0.01, 0.05 and 0.1 M), followed by freeze-drying. Protein contents of all acetic acid fractions were determined. The fractionation yields and protein recoveries of individual fractions as well as the gluten recovered from acetic acid fractions are shown in Tables 9.3 and 9.4, respectively.

The amount of gluten solubilised in acetic acid increased at higher acid concentration, demonstrated by the increasing yield of supernatants from 33.7 to 46.8 and 48.5% when gluten was fractionated with 0.01, 0.05 and 0.1 M acetic acid, respectively (Table 9.3). Correspondingly, the amounts of gluten remaining in the pellet fractions were reduced from 54.8 to 34.3 and 31.8% respectively with the increase of acetic acid from 0.01 to 0.05 and 0.1 M.

Table 9.3 Protein distributions in soluble and insoluble fractions

	Control	Soluble fraction (supernatant)			Insoluble fraction (pellet)		
Acetic acid concentration (M)		0.01	0.5	0.1	0.01	0.5	0.1
pH	-	3.4	3.1	2.9	-	-	-
Fractionation yield (%, w/w)	-	33.7	46.8	48.5	54.8	34.3	31.8
Protein content (%, w/w)	70.6	86.4	84.6	84.0	62.2	48.9	46.2
Protein recovery (%, w/w)	-	41.2	56.1	57.7	48.3	23.8	20.8

Table 9.4 Total yield and protein recovery from the fractionation of gluten at various acetic acid concentrations

	Gluten recovered from supernatants and pellets		
Acetic acid concentration	0.01 M	0.05 M	0.1 M
Total yield (%, w/w)	88.5	81.1	80.3
Overall protein recovery (%, w/w)	89.5	79.9	78.5

It was expected that gluten proteins would be solubilised in the acidic conditions, showing that protein contents of soluble fractions were higher than those of insoluble fractions (Table 9.3). The solubility of protein was higher at stronger acetic acid

concentrations, demonstrated by the increase of protein recovery in soluble fractions from 41.2 to 56.1 and 57.7% when the acetic acid concentration was increased from 0.01 to 0.05 and 0.1 M, respectively (Table 9.3). A similar trend of gluten protein solubilisation was also found in a previous study (Berot et al., 1994). It was observed that the level of protein recovery in the supernatant sharply increased between the 0.01 and 0.05 M acetic acid fractionation while there was only a small difference in the protein recovery of supernatants from the fractionation at 0.05 and 0.1 M acetic acid. This indicates that the effect of acetic acid on solubilising gluten protein is similar for both concentrations of 0.05 and 0.1 M.

Total yield and overall protein recovery of the fractionation at three different acetic acid concentrations (0.01, 0.05 and 0.1 M) were similar, ranging from 88.5 to 81.1 and 80.3 %, respectively for the yield and from 89.5 to 79.9 and 78.5 %, respectively for the protein recovery (Table 9.4). Although these values were below 100%, the yield and protein recovery were similar for all fractionations. This indicates that the loss of material has occurred evenly in all the treatments. Accordingly, the effect of acetic acid concentration on the distribution of protein and lipid within gluten fractions can be readily compared.

The protein profile of the gluten control and acetic acid treated gluten has been studied using reduced and non-reduced SDS-PAGE and SE-HPLC and these results are displayed in Figures 9.4 and 9.5. The majority of the proteins solubilised at the lower acetic acid concentration (0.01 M) had MW below 55 kDa (Figure 9.4, lane b) and were most likely gliadins and LMW-GS. There were also small amounts of glutenin being solubilised by 0.01 M acetic acid, demonstrated by several faint bands of HMW-GS on the reduced SDS-PAGE gel having MW above 66 kDa. These findings are consistent with the previous report on the extraction of gluten proteins with acetic acid (Berot et al., 1994), which found that the soluble fraction contained a very high content of protein solubilised in ethanol-water and a low content of HMW-GS.

Glutenins were more soluble at the high concentrations of acetic acid (0.05 and 0.1 M), demonstrated by the increased intensity of HMW-GS bands in the supernatant fractions (Figure 9.4, lane a, d and f). HMW-GS and LMW-GS associated in the gluten matrix through disulfide bonds, therefore they existed as polymeric molecules (Shewry et al.,

1992). Similar results were found in the fractionation of gluten with an increasing concentration of acetic acid (Berot et al., 1994) and hydrochloric acid (Cornec et al., 1994). The increase in solubility of gluten protein with the increasing acid concentration could be due to the affect of acid on hydrogen bonds within the gluten matrix, leads to the re-orientation of gluten structure (Wrigley et al., 2006). Furthermore, the pH of fractionation was from 2.9–3.4 whilst most amino acids of gluten proteins have pI values at 5.4–6.3. These conditions facilitate the formation of a net charge on the protein molecules, thereby promoting their solubilisation (Damodaran, 1996).

The predominance of monomeric protein in the 0.01 M soluble fraction and the increased solubility of glutenin in the supernatant with increasing acetic acid concentrations were also confirmed through SE-HPLC (Figure 9.5). These results showed that the 0.01 M acetic acid supernatant contained the highest ratio of monomeric to polymeric protein (2.3). This ratio decreased from 2.3 to 1.64 in the supernatant fractions when the concentrations of acetic acid used for the fractionations increased from 0.01 to 0.1 M, indicating the increase of polymeric protein content, particularly glutenin in the supernatant of the higher acetic acid concentrations.

In addition, protein bands with MW of approximately 50–55 kDa appeared on the non-reduced SDS-PAGE gel of the pellets but these were only faintly evident in that of the supernatants (Figure 9.4). This region of protein band was designated to ω-gliadins, and these are known to lack cysteine residues. They cannot form inter and intra-molecular disulfide bonds, are present as monomers and therefore are able to enter the non-reduced gel. These proteins can interact with other gluten components through hydrogen bonds and their presence in the glutenin-rich fraction of gluten has been reported previously (MacRitchie et al., 1991).

On the reduced SDS-PAGE, it was also observed that many protein bands with MW ranging from 27 to 12 kDa were present in the gluten control but varied in the gluten fractions (Figure 9.4). The protein bands at MW of approximately 27, 16 and 12–13 kDa were present in the soluble fractions, however the intensities of these bands were lower when increasing the acetic acid concentrations were used in fractionation. The reduction of band intensity could be a consequence of the increasing HMW-GS proportion in the soluble fraction of higher acetic acid concentrations.

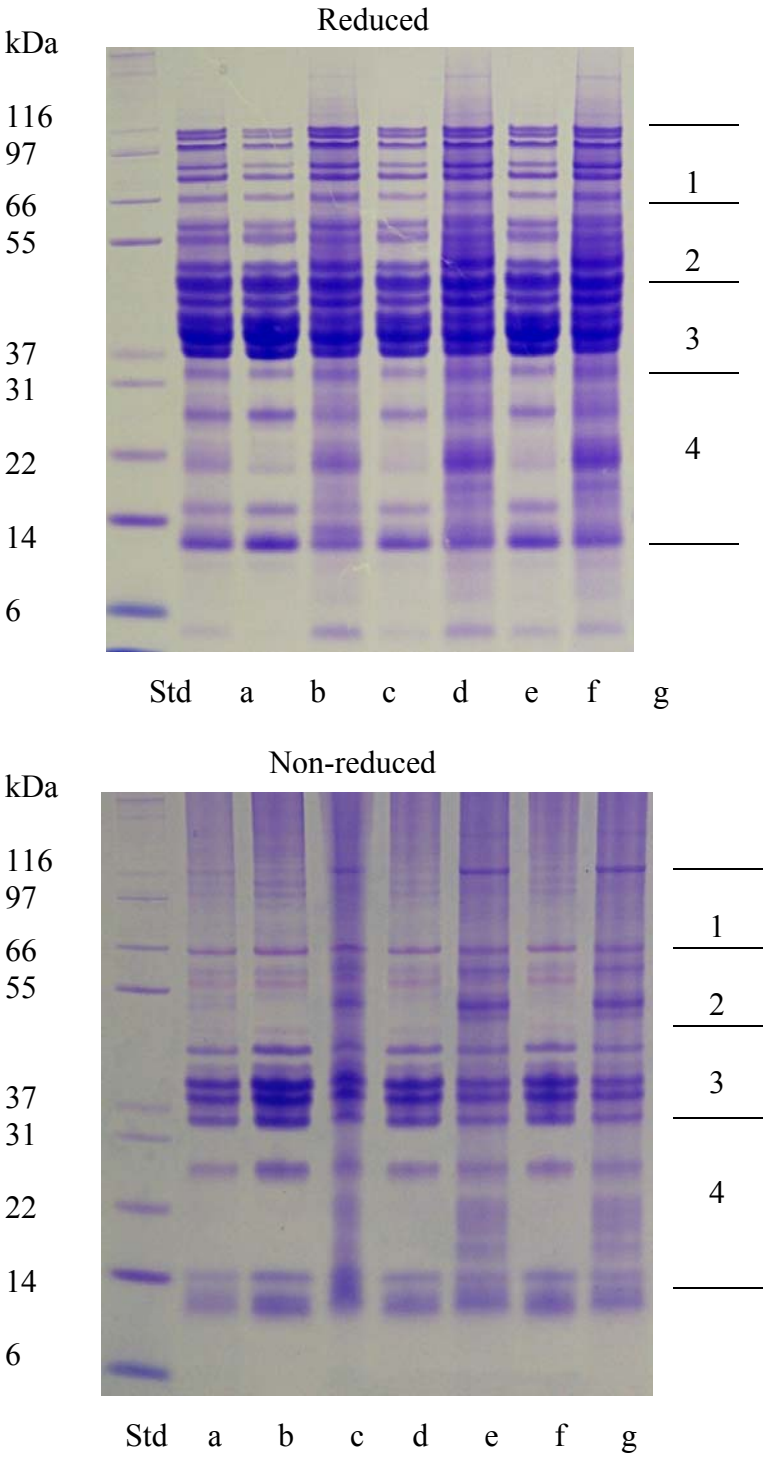


Figure 9.4 SDS-PAGE protein profiles of gluten and gluten fractions from acetic acid fractionation

Notes	a	Gluten control	std	Protein standard
	b,c	Supernatant and pellet fractions at 0.01 M acetic acid	1	Region of HMW-GS
	d,e	Supernatant and pellet fractions at 0.05 M acetic acid	2	Region of ω-gliadin
	f,g	Supernatant and pellet fractions at 0.1 M acetic acid	3 & 4	Region of α, γ, β-gliadin and LMW-GS

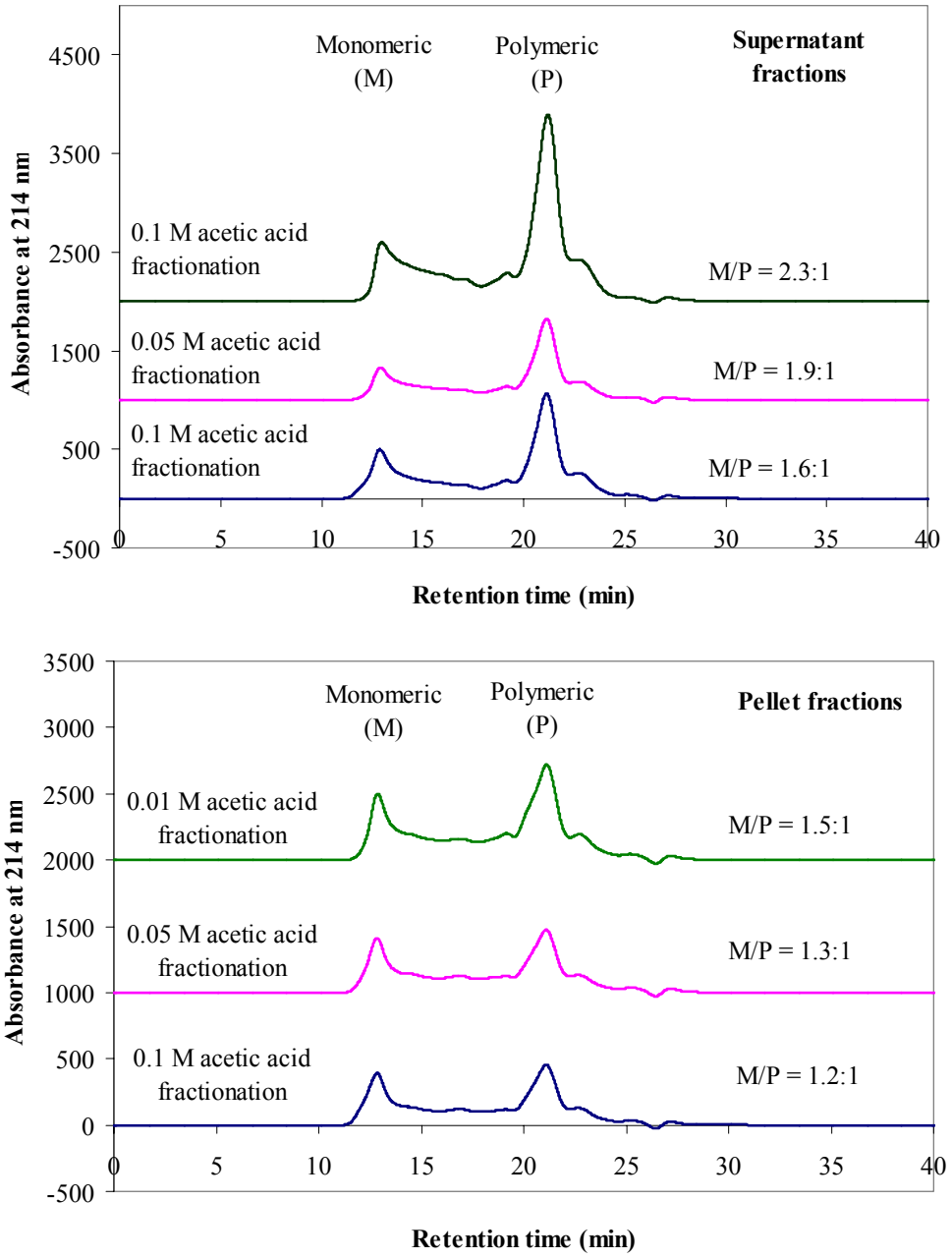


Figure 9.5 The SE-HPLC chromatograms of supernatants and pellets fractionated from gluten at selected acetic acid concentrations

9.3.2 Distribution of free and bound lipids and their lipid classes in acetic acid soluble and insoluble fractions

The free and bound lipid of the supernatant and pellet fractions were extracted and analysed for the lipid class composition on HPLC. In order to facilitate the comparison of lipid distribution within the fractions, the free and bound lipid contents were

calculated as the quantity of lipid in an individual fraction derived from 100 g of gluten. The lipid class composition of the free and bound lipid was presented as the proportion of lipid class in the corresponding lipid content. These values are shown in Table 9.5 for individual fractions and in Table 9.6 for the gluten recovered from the fractions.

Small amounts of free lipid were found in both the supernatant and pellet fractions and the distribution of this material was dependent on the concentration of acetic acid (Table 9.5). With increasing acetic acid concentrations, the levels of free lipid in the supernatants increased from 0.13 to 0.65% whilst those in pellets decreased from 0.47 to 0.21%. It was observed that this increase occurred primarily in the supernatants of 0.01 and 0.05 M acetic acid fractionation while the free lipid contents were similar for the supernatants of the 0.05 and 0.1 M acetic acid fractionations. Consequently, ratios of free lipid content between supernatants and pellets changed from 1:3 for the 0.01 M acetic acid fractionation to 3:1 for the 0.05 and 0.1 M acetic acid fractionations (Table 9.5).

Table 9.5 Free and bound lipid distributions in soluble and insoluble fractions

	Soluble fraction (supernatant)			**Insoluble fraction (pellet)**		
Acetic acid concentration (M)	0.01	0.05	0.1	0.01	0.05	0.1
Free lipid (%, w/w of gluten)	**0.13**	**0.65**	**0.65**	**0.47**	**0.21**	**0.24**
Non-polar lipids	(100)	(91)	(91)	(100)	(100)	(100)
Glycolipids	(0)	(9)	(9)	(0)	(0)	(0)
Phospholipids	(0)	(0)	(0)	(0)	(0)	(0)
Bound lipid (%, w/w of gluten)	**0.74**	**1.91**	**2.28**	**2.98**	**1.17**	**0.93**
Non-polar lipids	(31)	(32)	(35)	(45)	(48)	(40)
Glycolipids	(18)	(42)	(40)	(45)	(40)	(48)
Phospholipids	(51)	(26)	(25)	(10)	(12)	(12)

Notes: Free and bound lipid contents of supernatants and pellets are calculated based on 100 g of gluten used for fractionations and expressed as %, w/w of gluten
Non-polar lipids are TAG, DAG and MAG.
Glycolipids are MGDG and DGDG.
Phospholipids are PE, PI, PS, PC, LPE and LPC
Data in parentheses represent proportions expressed as a percentage of the lipid

Table 9.6 Total free and bound lipid in gluten recovered from fractionation at various acetic acid concentrations

	Gluten recovered from supernatants and pellets		
Acetic acid concentration (M)	0.01	0.05	0.1
Total lipid (%, w/w of gluten)	4.32	3.93	4.10
Total free lipid (%, w/w of gluten)	0.60	0.86	0.89
Total bound lipid (%, w/w of gluten)	3.72	3.07	3.21

Notes: Lipids in gluten recovered from the fractionations are a sum of lipid in supernatant and pellet calculated as %, w/w of gluten
Total lipid is calculated based on the amounts of free lipid and bound lipid.

For all of the fractionation experiments reported in this Section, supernatants and pellets were found to contain relatively high amounts of bound lipid (Table 9.5). The distribution of bound lipid in these fractions was similar to that of free lipid, with an increase of bound lipid levels in the supernatants and consequently a decrease of these lipids in the pellets when acetic acid of increasing concentration was used in the fractionation.

The amount of total free lipid was higher in the gluten recovered from acetic acid gluten fractions (0.60–0.89%, w/w, gluten) (Table 9.6) than in the gluten control (0.23%, w/w, gluten) (Table 9.2). Correspondingly, the amount of total bound lipid was lower in the gluten recovered from acetic acid gluten fractions than in the gluten control. These findings are consistent with the results of the acetic acid treated gluten (Section 9.2), which has been discussed in Section 9.2.2, indicating that changes of the gluten structure under acidic conditions have resulted in some bound lipids in gluten to now be extracted as free lipids.

Non-polar lipids were, as expected, found to be a major proportion of the free lipid extracts of both supernatant and pellet fractions (Table 9.5). Glycolipids were not found in the free lipid extract of the 0.01 M acetic acid supernatant, although a small amount of free glycolipids (0.06%, w/w, gluten) occurred in the supernatants following fractionation with higher acetic acid concentrations (0.05 and 0.1 M).

The relative proportions of the lipid classes in the bound lipid extracts from all pellets was similar, with higher amounts of both non-polar lipids and glycolipids and low levels of phospholipids (Table 9.5 and Figure 9.6). The bound lipid extracts of all supernatants contained a similar proportion of non-polar lipids but varied in glycolipids and phospholipids. The bound phospholipids occurred at a high level in the 0.01 M acetic acid supernatant whilst the proportion of bound glycolipids was high in the supernatant of the fractionation at higher acetic acid concentrations (0.05 and 0.1 M).

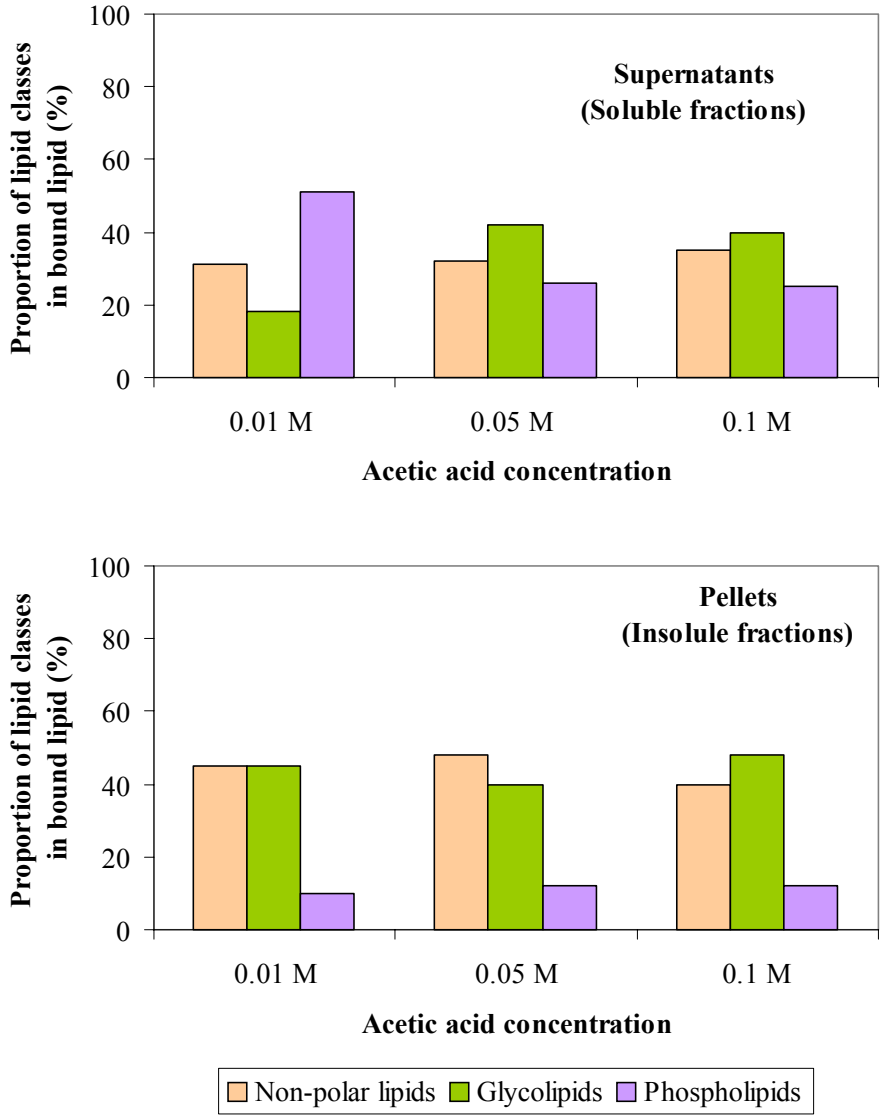


Figure 9.6 Distribution of lipid classes in bound lipid extracts from soluble and insoluble fractions

The distribution of free and bound lipid in the fractions indicates that a large proportion of free lipid remained with the insoluble fraction collected at the low acetic acid concentration (0.01 M) while a high amount of free lipid was co-extracted with the proteins soluble at the higher acetic concentrations (0.05 and 0.1 M). In the fractionation at low acetic acid concentration (0.01 M), when a proportion of gliadin was extracted into the supernatant, non-polar lipids held within gliadin and glutenin would remain on the surface of glutenin molecules in the pellets and can be extracted as free lipid. In the fractionation at higher acetic acid concentration (0.05 and 0.1 M), glutenin became more soluble and was present at a high proportion in the supernatants. Accordingly, these lipids remaining on the surface of glutenin were also extracted into the supernatant. This could be an explanation for the high amount of free non-polar lipid found in the supernatant of the fractionation at higher acetic acid concentrations.

It was observed that the amount of free lipid extracts and their lipid composition was similar in the supernatants of the fractionations at both concentration of acetic acid (0.05 and 0.1 M). Although the amount of bound lipids in these fractions differed slightly, their lipid composition was similar. This indicates that both concentrations of acetic acid (0.05 and 0.1 M) had the same effect on lipid distribution in the gluten fractions.

9.3.3 Lipid and protein distribution in relation to protein and lipid interaction

In order to observe the relationship between levels of protein and lipid extracted in these fractions, the amount of free and bound lipids in supernatants and pellets were plotted against the protein recovery and are shown in Figure 9.7. The amounts of free lipids in both supernatants and pellets increase slightly with the increase of protein extracted in these fractions. In addition, the amounts of these lipids per g of protein were similar for almost all the supernatants and pellets fractionated at various acetic acid concentrations (0.01, 0.05 and 0.1 M), with the exception of the supernatant from the 0.01 M acetic acid fractionation, which contained a relatively lower amount of lipid (Figure 9.8). The differences in the level of free lipid in supernatants and pellets were too small compared to the large range of protein recovery in these fractions. These results indicate that the free lipid content in both acetic acid soluble and insoluble fractions was not proportionally related to the level of protein recovered in these fractions.

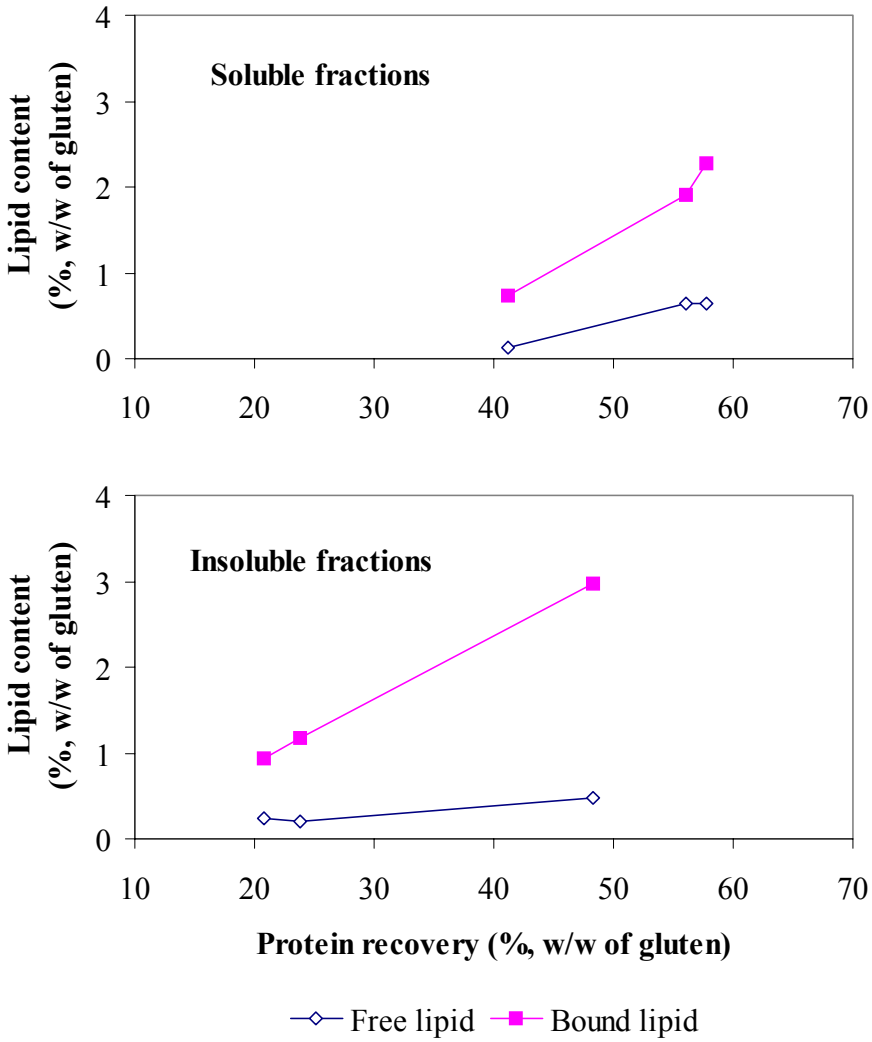


Figure 9.7 Relationship of free and bound lipid level with protein recovery in soluble and insoluble fractions

The amount of total bound lipid in the supernatant and pellet fractions increased proportionally with the increased amount of protein extracted into these fractions (Figure 9.7). Furthermore, the amount of bound lipid per g of protein increased in the supernatants fractionated at increasing concentrations of acetic acid and consequently these values decreased in the pellets (Figure 9.8). This indicates that those lipids found at increasing levels in the supernatants are preferentially associating with the proteins that become more soluble in supernatants when the fractionation of gluten was carried out at higher acetic acid concentrations.

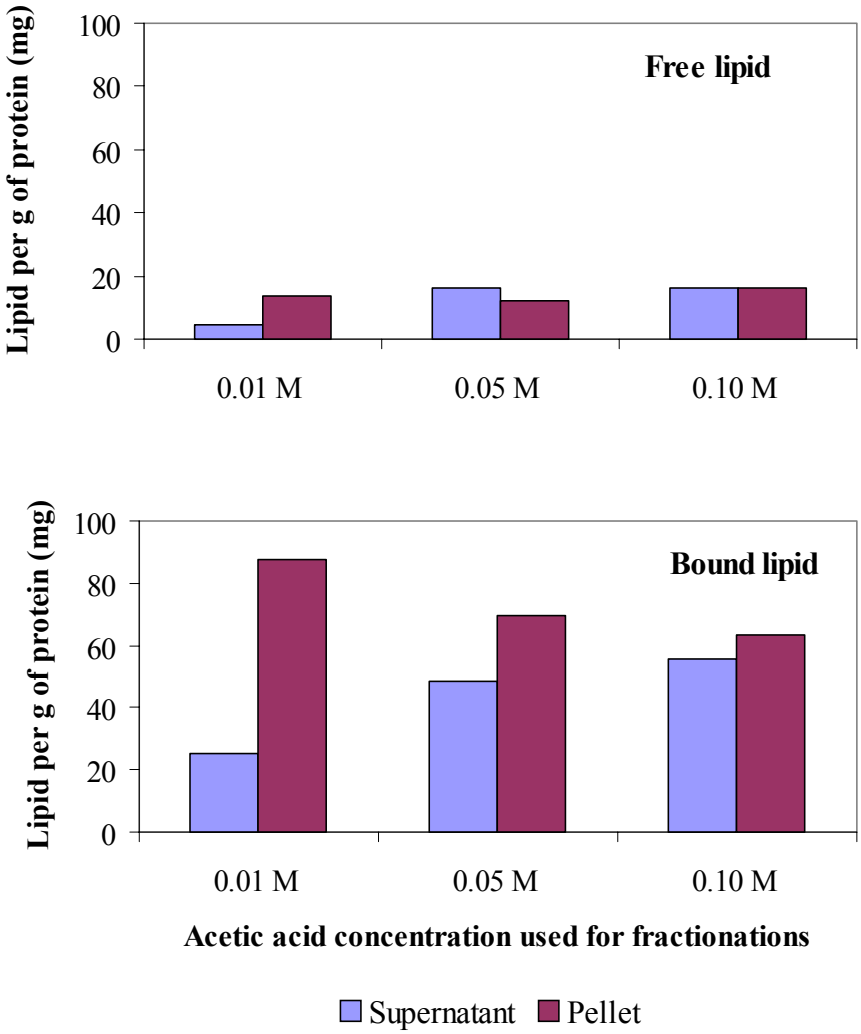


Figure 9.8 The association of free and bound lipid with protein in soluble and insoluble fractions

The results from protein analysis showed that glutenins were more soluble at higher acetic acid concentrations (Section 9.3.1). Increased amounts of bound non-polar lipid and glycolipid were present when the protein content increased in the supernatant fractions corresponding with the use of acetic acid at higher concentrations for fractionation (Figure 9.9, B,D). This observation indicates that these lipids were likely to be associated with the polymeric glutenin. It is well known that in the gluten matrix HMW-GS are present in the polymeric form and involve both inter and intra-molecular disulfide covalent bonds (Shewry et al., 1992). The lowest of the three different concentrations of acetic acid used in this experiment did not appear to have an effect on

the disulfide bonds in these proteins. The non-polar lipids and glycolipid co-existed with the proteins that were solubilised at higher acetic acid concentrations (0.05 and 0.1 M) could be present as a protein-lipid complex in the soluble fractions.

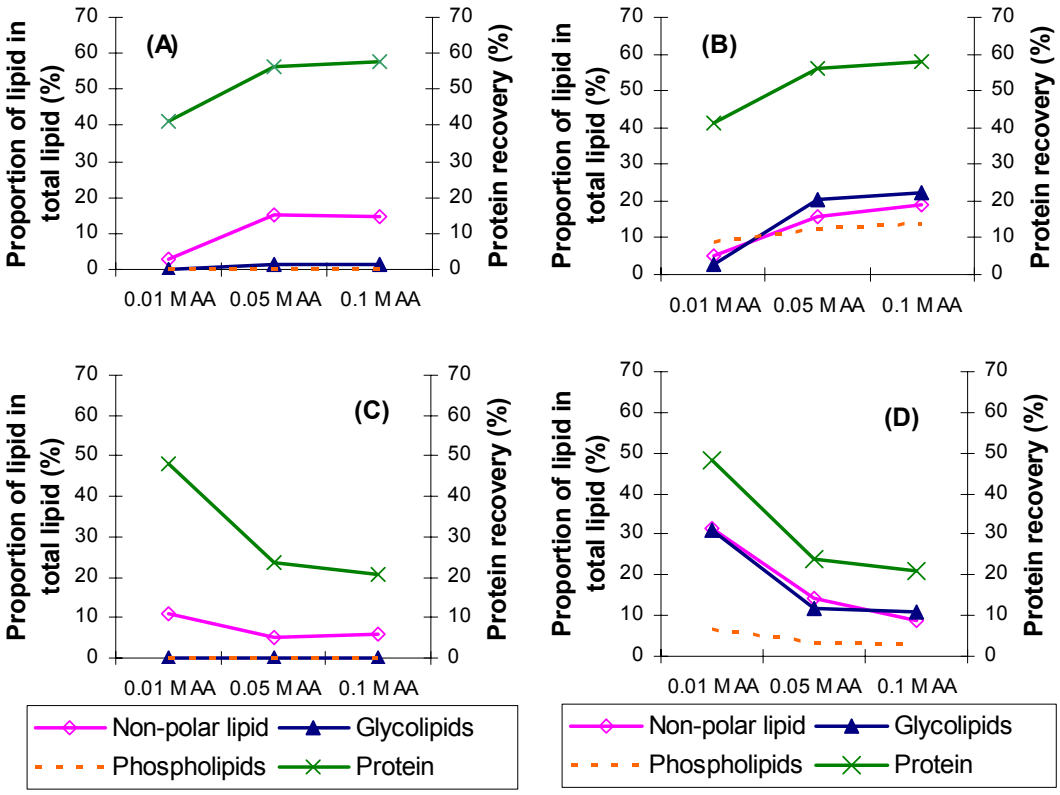


Figure 9.9 Distribution of lipid classes as free and bound lipids in supernatant and pellet fractions

Notes
A Free lipid in supernatant
B Bound lipid in supernatant
C Free lipid in pellet
D Bound lipid in pellet
AA represents acetic acid

The distribution of bound phospholipid did not relate directly to the level of protein recovery in gluten fractions (Figure 9.9, B,D). This indicates that the glutenin, which became more soluble at increasing acetic acid concentrations, does not interact with phospholipids. Moreover, the relatively high level of phospholipid as well as the high proportion of monomeric proteins in the 0.01 M acetic acid supernatant suggests that phospholipid preferentially associates with monomeric proteins.

9.4 General discussion and summary

Acetic acid showed an effect on the dissociation of lipids from the gluten matrix. This could be due to acetic acid disrupting the hydrogen bonds between glutenin and gliadin, allowing some lipids to be accessible to the non-polar solvent during extraction. It is likely that the non-polar lipid is associated with gluten proteins through hydrophobic bonds; therefore, the extraction of higher levels of free lipids upon treatment with acetic acid particularly affected non-polar lipids rather than the polar lipids including glycolipids and phospholipids.

Fractionation of gluten protein at various acetic concentrations indicated that non-polar lipid and glycolipid are probably associated with glutenin as a complex, which becomes more soluble at the higher acetic acid concentrations (0.05 and 0.1 M). It was also observed that the phospholipids were preferentially extracted with the monomeric proteins, indicating the interaction of phospholipids with these proteins. Differences in lipid and protein distribution were clearly observed between 0.01 and 0.05 M acetic acid fractionations but not between those involving 0.05 and 0.1 M acetic acid.

Chapter 10

Results and discussion: Investigation of protein and lipid interactions using sequential acetic acid fractionation

10.1 Introduction

The results described in Chapter 9 demonstrate that treating gluten with acetic acid has altered the extractability of lipids from the gluten matrix. Firstly, more lipids became extractable with petroleum ether and this is attributed to the effect of acetic acid on the gluten structure. Secondly, a single fractionation of gluten with various concentrations of acetic acid produced protein fractions having different proportions of monomeric and polymeric proteins. These results indicate that non-polar lipid and glycolipid might be associated with glutenin as a protein-lipid complex, and that the phospholipids may preferentially interact with monomeric proteins.

However, the use of simple fractionation steps using solutions of acetic acid of particular concentrations does not clearly show the effect of increasing acetic acid concentration on the distribution of protein and lipid components. The soluble fraction at a higher acetic acid concentration can contain the protein solubilised at both lower and higher acetic acid concentrations. Previously, it has been reported that sequential fractionation of gluten with hydrochloric acid solutions at different concentrations produced protein fractions having various gliadin-to-glutenin ratios (MacRitchie, 1985; Cornec et al., 1994). In the current study (Chapter 9), 0.05 and 0.1 M acetic acid both had a similar effect on the protein and lipid distribution. In this chapter, the sequential fractionation with 0.01 and 0.1 M acetic acid was applied to further investigate the effect of acetic acid on protein and lipid distribution in gluten fractions in order to provide an enhanced understanding of the interactions of protein and lipid in gluten.

Gluten (petroleum ether defatted gluten) was fractionated using the procedure described in Section 7.3. Following fractionation, lipid and protein were analysed using the procedures in Sections 7.5 and 7.6, respectively, for all fractionated samples along with

the gluten control. The distribution of protein and lipid within the fractions was examined and interpreted in order to elucidate the protein-lipid interactions in gluten.

10.2 Protein distribution in sequential acetic acid gluten fractions

The gluten control sample was fractionated by firstly using acetic acid of relatively low concentration (0.01 M). The insoluble residue from this extraction was then extracted with a higher acetic acid concentration (0.1 M). This provided three fractions: the 0.01 M acetic acid soluble fraction, 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction. The results obtained are presented in Table 10.1. The three fractions gave a total yield of 95% and approximately one third of the original gluten was recovered in each of the three fractions.

Table 10.1 Protein in gluten control and gluten fractions

	Gluten control	Sequential acetic acid fractionation			
		0.01M acetic acid soluble fraction	0.1M acetic acid soluble fraction	Insoluble fraction	Total
Fractionation yield (%)	-	27.3 ± 0.1^{a}	33.7 ± 0.6^{b}	34.3 ± 0.2^{b}	95.3 ± 0.6
Protein content (%)	71.6 ± 0.2	90.2 ± 0.4^{a}	83.2 ± 0.8^{a}	48.0 ± 0.4^{b}	-
Protein recovery (% of gluten)	-	34.3 ± 0.1^{a}	39.3 ± 0.7^{b}	23.0 ± 0.2^{c}	96.6 ± 0.6

Notes: Superscript letters are used to show statistically significant differences between means within the same row ($p<0.01$): values followed by the same letter are not significantly different
Means are calculated based on the three replicates

Although the amount of gluten was distributed reasonably evenly among the three fractions, the protein contents differed. Both 0.01 and 0.1 M acetic acid soluble fractions contained high amounts of protein, with values of 90.2 and 83.2% (w/w) respectively, while only 48% (w/w) protein was found in the insoluble fraction (Table 10.1). This result could be predicted since the non-protein matter (particularly starch and fibre residues) in gluten would remain as the insoluble material through the

fractionation process. In addition, the higher amounts of protein were recovered in the 0.01 and 0.1 M acetic acid soluble fractions at the level of 34.3 and 39.3% of the total gluten protein, respectively. Consequently, only 23.0% of the total gluten protein remained in the insoluble fraction. These values of protein recovered in each fraction were summed to give the total protein recovery following fractionation, reaching 96.6% (Table 10.1).

Similar levels of protein were recovered in the two acetic acid soluble fractions; however, the distribution of monomeric and polymeric proteins in those fractions was different as determined by SE-HPLC (Figure 10.1). This reflected the effect of sequential fractionation with two different acetic acid concentrations on solubilising different types of gluten protein. Results of SE-HPLC showed that the ratio of monomeric-to-polymeric proteins was higher in the 0.01 M acetic acid soluble fraction (ratio of 2.40) than in the 0.1 M acetic acid soluble fractions (ratio of 1.46) and gluten control (ratio of 1.71) (Figure 10.1). The first soluble fraction contained a high content of monomeric protein, while the second soluble fraction had a high content of polymeric protein. The increasing amount of polymeric content in the second gluten extraction has been reported in previous studies on the fractionation of gluten using diluted acetic acid (Berot et al., 1994) and hydrochloric acid (Cornec et al., 1994). The increased rate of solubilisation of polymeric protein in the 0.1 M soluble fraction could be attributed to the effect of an increasing level of acetic acid and the additional mixing on the gluten structure during the second fractionation.

The insoluble fraction was also relatively rich in polymeric proteins, based upon the further reduction in the ratio of the monomeric-to-polymeric proteins as determined by SE-HPLC (Figure 10.1). The highest proportion of polymeric protein was in the insoluble residue with a ratio of 1.05. Much of the protein in the pellet was polymeric protein, formed between HMW-GS and LMW-GS through the disulfide bonds (Shewry et al., 1992). The lower protein recovery in the pellet indicates that a considerable proportion of the original polymeric protein is present in the acetic acid extracts. However, the limited extractability indicates that the polymeric protein in the pellet probably has a greater MW distribution than the extractable polymeric protein.

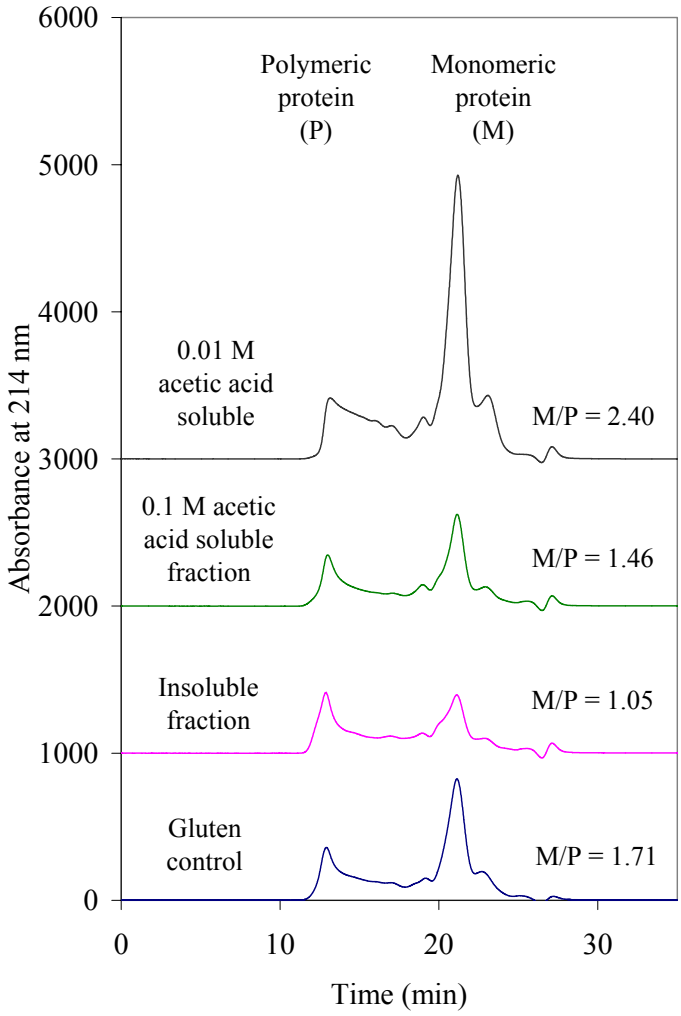


Figure 10.1 SE-HPLC pattern of proteins in gluten control and sequential acetic acid fractions

The increased proportion of polymeric proteins in the second soluble fraction and the acetic acid insoluble fraction was also confirmed through the further characterisation of the three gluten fractions using reducing and non-reducing SDS-PAGE gels. The results of SDS-PAGE were also used to verify the composition of protein types in each fraction.

The appearance of gliadin bands on the non-reduced gel for the 0.01 and 0.1 M acetic acid soluble fractions (Figure 10.2, right gel, lanes a and b) indicates the presence of monomeric proteins in these fractions. In contrast, the presence of glutenin subunits in the reduced gel indicates the presence of polymeric protein in the original preparations.

Protein bands in the region of HMW-GS on the reduced gel appear very weak in the 0.01 M acetic acid soluble fraction (Figure 10.2, left gel, lane a), while they are more intense in the 0.1 M acetic acid soluble and insoluble fractions (Figure 10.2, left gel, lanes b and c). This indicates that gliadins dominate in the 0.01 M acetic acid soluble fraction while large amounts of glutenin were solubilised in 0.1 M acetic acid soluble fraction (Figure 10.2).

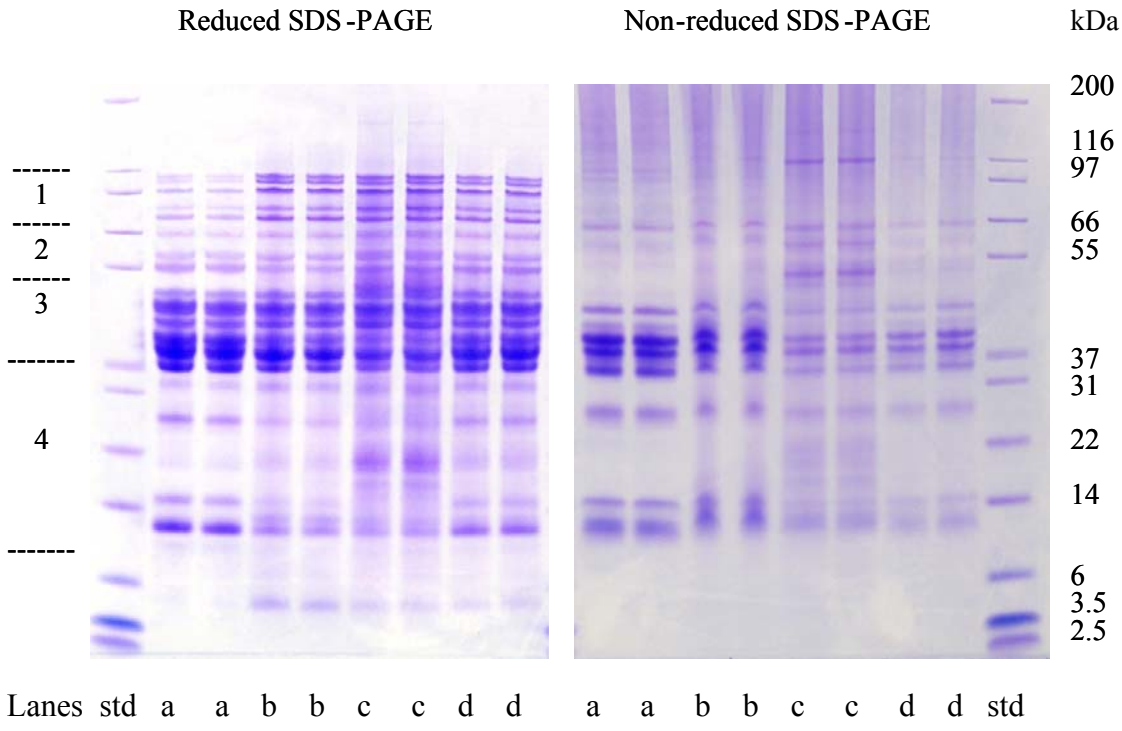


Figure 10.2 Reduced and non-reduced SDS-PAGE protein pattern of gluten control and sequential acetic acid fractions

Notes
- a 0.01 M acetic acid soluble fraction
- b 0.1 M acetic acid soluble fraction
- c Insoluble fraction
- d Gluten control
- Std Protein standard
- 1 Region of HMW-GS
- 2 Region of ω-gliadins
- 3 &4 Region of α,β,γ-gliadins and LMW-GS

Some faint protein bands are found in the region of 30–55 kDa in the insoluble fraction under the non-reduced condition (Figure 10.2, right gel, lane c), indicating that a small amount of monomeric gliadin was present in this fraction. However, under the reduced condition, the strong intensity of protein bands was found in the same MW range of

30–55 kDa, a region of LMW-GS and α,β,γ-gliadins, as well as many protein bands appeared in the HMW-GS region. This could reflect the presence of a high proportion of polymeric glutenin in this fraction.

The differences of protein distribution in the three gluten fractions were investigated quantitatively by comparison of protein bands on the densitogram of the reduced SDS-PAGE gel (Figure 10.3). The protein pattern of the 0.01 M acetic acid soluble fraction was clearly different to that of the other two gluten fractions, which have very similar protein patterns (Figure 10.3). In the MW range of 66–116 kDa, many high-density peaks dominated the patterns for both the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction, while low-intensity peaks were present in the 0.01 M acetic acid soluble fraction. In the MW range of 6–55 kDa, a number of peaks (12, 16, 26.8 and 42 kDa) appeared at high intensity in the 0.01 M acetic acid soluble fraction but were weak or not present in the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction.

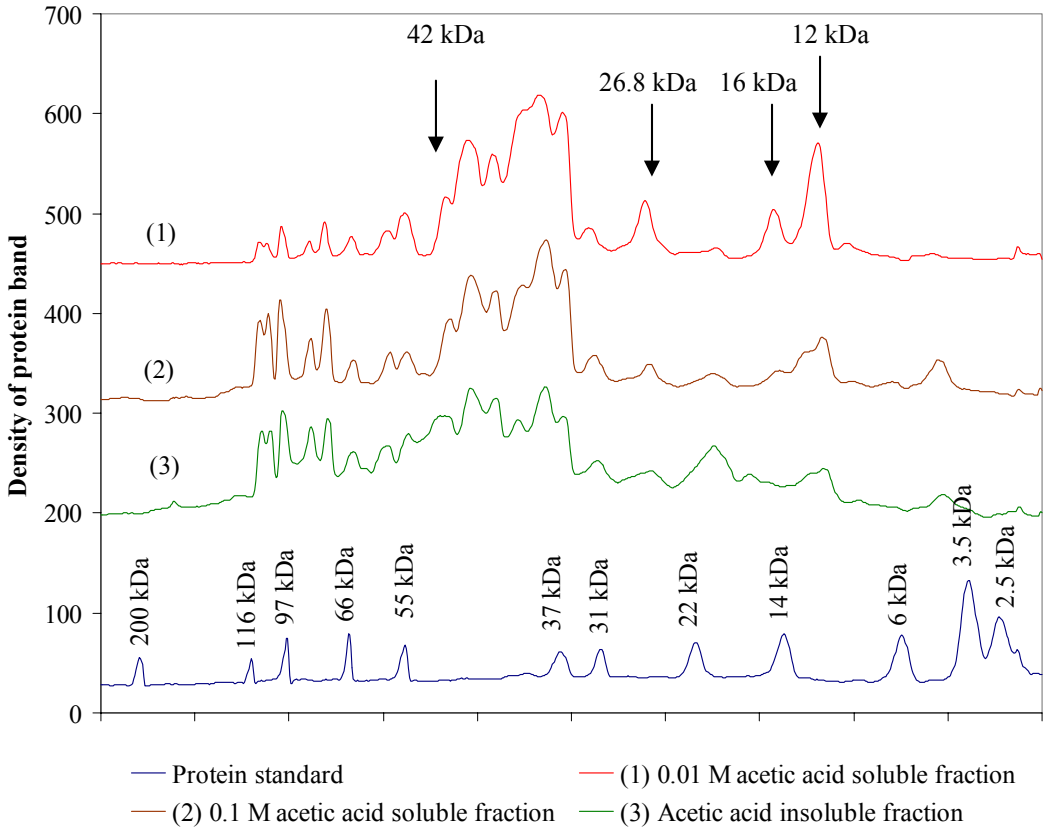


Figure 10.3 Comparison of protein band density on the reduced SDS-PAGE of sequential acetic acid fractions

In summary, the sequential fractionation with solutions of increasing concentrations of acetic acid was effective in separating gluten proteins into three fractions having different protein patterns. The 0.01 M acetic acid soluble fraction was predominantly monomeric protein, particularly gliadins, while the 0.1 M acetic acid soluble fraction contained both gliadins and glutenin, as glutenin was solubilised more fully during the second extraction with the increased acetic acid concentration. Polymeric glutenin was the major protein in the acetic acid insoluble fraction. Apart from protein, the insoluble fraction also contained a considerable amount of non-protein matter, probably starch and fibre. The proteins with MW of approximately 12, 16, 26.8 and 42 kDa were found predominantly in the 0.01 M acetic acid soluble fraction with relatively little in the other two fractions. Accordingly, the lipid content and the relative proportions of the individual lipid classes in these fractions warrant further investigation in order to establish if there is a relationship with the protein distribution.

10.3 Lipid content and extractability after the sequential acetic acid fractionation

Prior to studying the distribution of lipid components in the sequential acetic acid fractions, the total lipid content of the gluten control was compared with the sum of lipid composition found in the three fractions obtained from the sequential extraction of gluten. The purpose of this experiment was to establish whether there is any alteration in the lipids during sequential fractionation. The extractability of lipid as free and bound lipids from the gluten control was also compared with the sum from the sequential fractions to determine the effect of acetic acid on the interaction of lipid with other gluten components. In order to facilitate the comparison, the amounts of lipid in the gluten control and sequential acetic acid fractions are expressed in relation to 100 g of gluten control. When the amount of lipid was summed for the three fractions obtained from the gluten, these were calculated as the total amount of lipid obtained during fractionation of 100 g of the original defatted gluten control sample.

The results of these comparisons are presented in Table 10.2. These show that the total lipid content was not significantly different ($P = 0.073$) between the gluten control and the sum from the sequential acetic acid fractionation. In relation to the groups of lipid

classes, there were differences in the amounts of total non-polar lipid and glycolipids (P = 0.017 and 0.018, respectively) but the difference was not observed for phospholipids (P = 0.073) (Table 10.3). Of the eleven lipid classes considered in this study, not all showed differences; however, there were significant increases in DAG and LPC (P<0.01) and decreases in MGDG (P = 0.01) and PI (P = 0.02). The changes in the amounts of these lipids after the acetic acid fractionation might be due to the effect of acetic acid on the interaction of these lipids with other gluten components or alternatively result from hydrolysis of other lipids, particularly glycolipids. The DAG and LPC represent a relatively small amount in the gluten control. It is possible that slight changes could cause a significant difference in those values whilst not significantly influencing the value for total lipid content. The results indicate that the sequential acetic acid fractionation did not alter the total amount of lipid but did impact on some of the individual components as well as the lipid classes in gluten.

Table 10.2 Comparison of free, bound and total lipids in the gluten control and the sum for the fractions from the sequential fractionation

	Gluten control	Sum for the sequential fractionation	Probability of similarity (ANOVA)
	(g of lipid in 100 g gluten)		
Free lipids	0.19 ± 0.05	1.34 ± 0.13	<0.01
Bound lipids	4.55 ± 0.16	3.56 ± 0.11	<0.01
Total lipids	4.74 ± 0.06	4.90 ± 0.02	0.073

Notes: Total lipid values have been calculated from the amounts of free lipid and bound lipid.

The sequential acetic acid fractionation did not affect the total amount of lipid extract, but the level of free and bound lipid in gluten changed (Table 10.2). This was demonstrated by a significant increase (P <0.01) of the free lipid content in the sum of gluten from the sequential fractionation (1.34 g) compared to the gluten control (0.14 g). Consequently, the bound lipid content was significantly lower in the gluten fractions (in total, 3.56 g) than in the gluten control (4.74 g). As the gluten control had been defatted using petroleum ether, the level of free lipid in the gluten control was relatively low, representing approximately 4% of the total lipid. Following fractionation with

acetic acid, the content of free lipid increased to approximately 27% of the total lipid. This increase confirms the results reported in Chapter 9 in relation to the effect of acetic acid on the extractability of lipid from the gluten matrix. It is noted that the two experiments were conducted on two different batches of gluten (similarly prepared), and this is probably the reason for the differences of total lipid in the gluten control samples (5.7, see Section 9.2 compared to 4.74, Table 10.2).

Table 10.3 Comparison of lipid classes in gluten control and recovered from sequential acetic acid fractionation as total lipid

	Gluten control	Sum for the sequential fractionation	Probability of similarity (ANOVA)
	(mg of lipid in 100 g gluten)		
Total non-polar lipids	**1845 ± 81**	**2083 ± 67**	**0.017**
TAG	1344 ± 70	1467 ± 56	0.077
DAG	135 ± 1	222 ± 31	< 0.01
MAG	364 ± 10	393 ± 36	0.263
Total glycolipids	**2100 ± 80**	**1901 ± 39**	**0.018**
MGDG	1596 ± 62	1421 ± 20	0.010
DGDG	504 ± 22	479 ± 20	0.293
Total phospholipids	**791 ± 102**	**917 ± 76**	**0.073**
PE	19 ± 2	14 ± 9	0.446
PI	20 ± 1	16 ± 1	0.020
PS + PC	545 ± 60	517 ± 30	0.509
LPE	93 ± 16	124 ± 16	0.033
LPC	114 ± 24	244 ± 32	< 0.01

Notes The values for the non-polar lipids, glycolipids and phospholipids were calculated as the sum of the contents of each of the individual components measured by HPLC
Abbreviations used are:

TAG	Triacylglycerol	PI	Phosphatidylinositol
DAG	Diacylglycerol	PS	Phosphatidylserine
MAG	Monoacylglycerol	PC	Phosphatidylcholine
MDDG	Monogalactosyl diglyceride	LPE	Lysophosphatidylethanolamine
DGDG	Digalactosyl diglyceride	LPC	Lysophosphatidylcholine
PE	Phosphatidylethanolamine		

The change in the level of free and bound lipids in gluten after the sequential acetic acid fractionation can be also observed at the level of the lipid classes (Table 10.4). In comparison to the gluten control, there was a significant increase ($P<0.01$) in TAG, DAG, MAG, MGDG and DGDG as free lipid and consequently, a significant corresponding decrease ($P<0.01$) of those lipids found as bound lipid, except in the case of MAG ($P = 0.03$).

Most of phospholipids occurred as bound lipid in the gluten control and also following sequential fractionation. Very small amounts of PE and PS + PC (76 mg in 100 g of gluten) were found as free lipid following sequential fractionation. Of the bound lipid, the amounts of phospholipids including PE and PS + PC were not found to be significantly different between the gluten control and the sum from sequential fractionation (Table 10.4). The amount of bound LPC was significantly higher ($P<0.01$) in the gluten from sequential fractionation (244 mg) than in the gluten control (114 mg). This could be related to the increased amount of LPC in the sum of lipids following sequential fractionation.

In summary, the results from the lipid analyses showed that there were no significant differences in the total lipid content and total lipid classes between the gluten control and the sum following sequential fractionation. This indicated that the sequential acetic acid fractionation did not cause any significant changes in the levels of the total lipid extract. However, the contents of non-polar lipids, glycolipids and phospholipids have changed. The sequential acetic acid fractionation did not have an effect on the total amount of most of specific lipid classes including TAG, MAG, DGDG, PE, and PS + PC, except for DAG, LPE and LPC that became more extractable after the acetic acid fractionation. It has been observed that levels of free and bound lipid in the gluten changed after sequential acetic acid fractionation. The increase in the amount of free lipid and the decrease of amount of bound lipid in the gluten after the fractionation confirmed the effect of acetic acid on the dissociation of lipid from the gluten matrix.

Table 10.4 Comparison of lipid classes in gluten control and recovered from sequential acetic acid fractionation as free and bound lipids

	Control gluten	Sum for the sequential fractionation	Probability of similarity (ANOVA)
	(mg of lipid in 100 g gluten)		
Free lipid			
TAG	139 ± 43	687 ± 111	<0.01
DAG	5 ± 1	144 ± 18	<0.01
MAG	42 ± 10	126 ± 11	<0.01
MGDG	0.5 ± 0.9	228 ± 22	<0.01
DGDG	0	83 ± 4	<0.01
PE, PS + PC	0	76 ± 3	<0.01
Bound lipid			
TAG	1205 ± 45	781 ± 55	<0.01
DAG	131 ± 1	78 ± 13	<0.01
MAG	322 ± 11	267 ± 28	0.03
MGDG	1595 ± 62	1193 ± 32	<0.01
DGDG	504 ± 22	397 ± 24	<0.01
PE	18 ± 2	11 ± 7	0.18
PI	20 ± 1	16 ± 1	0.02
PS + PC	545 ± 60	444 ± 31	0.06
LPE	93 ± 16	124 ± 6	0.03
LPC	114 ± 24	244 ± 32	<0.01

Notes: Abbreviations used are:

TAG	Triacylglycerol	PI	Phosphatidylinositol
DAG	Diacylglycerol	PS	Phosphatidylserine
MAG	Monoacylglycerol	PC	Phosphatidylcholine
MDDG	Monogalactosyl diglyceride	LPE	Lysophosphatidylethanolamine
DGDG	Digalactosyl diglyceride	LPC	Lysophosphatidylcholine
PE	Phosphatidylethanolamine		

10.4 Distribution of lipid in the sequential acetic acid fractions

10.4.1 Total lipid, free lipid and bound lipid distributions

Upon fractionation using acetic acid solutions, the amount of lipid in the gluten control was distributed at various proportions into the three fractions (Table 10.5). The level of lipid was significantly higher ($P<0.01$) in the 0.1 M acetic acid soluble fraction and reduced in the order of the acetic acid insoluble fraction and the 0.01 M acetic acid soluble fraction. Of the total lipid content, approximately 60% was extracted into the second soluble fraction (2.87 g in 100 g of gluten, Table 10.5), only 10% went into the first soluble fraction (0.61) and 30% remained in the pellet (1.42).

The proportion of free and bound lipids was different in the three fractions (Table 10.5). Of the total lipid in each fraction, the 0.01 M acetic acid soluble fraction contained 10% of the free lipid and 90% of the bound lipid, these amounts being close to the proportions of free and bound lipid in the gluten control (4 and 96% respectively). High proportions of free lipid and low proportions of bound lipid were found in the 0.1 M acetic acid soluble fraction (35 and 65% respectively) and in the acetic acid insoluble fraction (20 and 80%, respectively). This indicates that, although relatively little lipid was present, most of the lipid in the 0.01 M acetic acid soluble fraction was associated with other components in the fraction. The 0.1 M acetic acid soluble fraction contained the highest amount of lipid; however, nearly half occurred in the form that was readily extractable with petroleum ether.

In a comparison of the distribution of free and bound lipids obtained from a single fractionation (Table 9.5), the proportions of free and bound lipid in the acetic acid insoluble fraction were similar to those in the pellet of a single 0.1 M acetic acid fractionation shown in Table 9.5. This indicates that the proportions of free and bound lipid in the sum of both 0.01 and 0.1 M acetic acid soluble fractions are equal to those in the supernatant of the single 0.1 M acetic acid fractionation. As the proportions of free and bound lipid in the 0.01 M acetic acid soluble fraction were close to those in the supernatant of a single 0.01 M acetic acid fractionation (Table 9.5), the distribution of free and bound lipid in the 0.1 M acetic acid soluble fractions could be similar to the

difference of free and bound lipid level between two supernatants obtained from single fractions at 0.01 and 0.1 M acetic acid.

Table 10.5 Distribution of total non-polar lipids, glycolipids and phospholipids in sequential extraction fractions

	Sequential acetic acid fraction		
	0.01 M acetic acid soluble fraction	**0.1 M acetic acid soluble fraction**	**Insoluble fraction**
Total lipid (%, w/w gluten)	0.61 ± 0.04^{a}	2.87 ± 0.11^{b}	1.42 ± 0.09^{c}
Free lipid (%)	(10)	(35)	(20)
Bound lipid (%)	(90)	(65)	(80)
Free lipid (%, w/w gluten)	0.06 ± 0.01^{a}	1.00 ± 0.02^{b}	0.29 ± 0.13^{c}
Non-polar lipids (%)	(100)	(61)	(100)
Glycolipids (%)	(0)	(31)	(0)
Phospholipid (%)	(0)	(8)	(0)
Bound lipid (%, w/w gluten)	0.55 ± 0.05^{a}	1.88 ± 0.09^{b}	1.13 ± 0.07^{c}
Non-polar lipids (%)	(15)	(33)	(37)
Glycolipids (%)	(19)	(51)	(47)
Phospholipid (%)	(66)	(16)	(16)

Notes: Total lipid was calculated based on the amounts of free lipid and bound lipid.
Non-polar lipids are TAG, DAG and MAG.
Glycolipids are MGDG and DGDG.
Phospholipids are PE, PI, PS, PC, LPE and LPC
Data in parentheses represent proportions expressed as a percentage of the lipid class
Superscript letters are used to show statistically significant differences between means within the same row ($p<0.01$): values followed by the same letter are not significantly different

Based on the results presented in Chapter 9, both of the acetic acid concentrations (0.01 and 0.1 M) had the similar effect of increasing the free lipid content in gluten. The low amount of free lipid in the 0.01 M acetic acid soluble fraction is consistent with the result of a single fractionation at 0.01 M acetic acid (Section 9.3.2), in which most of the free lipids remained in the pellet after the first fractionation. At the second

fractionation with a higher acetic acid concentration (0.1 M), the free lipid retained in the pellet was recovered in the 0.1 M acetic acid soluble fraction.

The distribution of free lipid within the three gluten fractions was significantly different ($P<0.01$) due to the effect of the acetic acid during the fractionation steps (Table 10.5). Of the total amount of free lipid (1.35 g in the 100 g of gluten from the sequential fractionation), the 0.1 M acetic acid soluble fraction contained the highest proportion (75%), then the acetic acid insoluble fraction (21%) and the lowest level (4%) was found in the 0.01 M acetic acid soluble fraction. This indicates that the amount of lipid that was rendered extractable with petroleum ether after fractionating with acetic acid was not present in the 0.01 M acetic acid soluble fraction nor retained in the pellet fraction. Most was present in the 0.1 M acetic acid soluble fraction. The distribution of the three main lipid groups, non-polar lipid, glycolipid and phospholipid, was also different in the fractions obtained from the gluten. Non-polar lipid was a major lipid component in the free lipid of both the 0.01 M acetic acid soluble fraction and acetic acid insoluble fraction. This is similar to the observations for the gluten control, while the free lipid of the 0.1 M acetic acid soluble fraction consisted of each of the groups of lipid: non-polar lipid (61%), glycolipids (31%) and a small amount of phospholipid (8%) (Table 10.5).

The bound lipid was distributed within the three sequential fractions at significantly different amounts ($P<0.01$) with a similar overall pattern in the free lipid distribution (Table 10.5). The highest amount of bound lipid (expressed as g in 100 g gluten) was found in the 0.1 M acetic acid soluble fraction (1.88), followed by the acetic acid insoluble fraction (1.13) and the lowest was in the 0.01 M acetic acid soluble fraction (0.55). The three main groups of lipid classes were all present in the bound lipid extract from sequential fractionation, which was consistent with the bound lipid of the gluten control (Table 10.4). The pattern of distribution of the three main lipid groups in the bound lipid of the sequential fractions was particularly interesting. While the bound lipid of the 0.1 M acetic acid soluble and acetic acid insoluble fraction were similar, the 0.01 M acetic acid soluble fraction was quite different (Table 10.5 and Figure 10.4). The bound lipid of the first two fractions contained the highest proportion of glycolipid, a medium amount in non-polar lipid and the lowest proportion of phospholipid. On the other hand, the 0.01 M acetic acid soluble fraction had the highest proportion of

phospholipid and the lowest proportions of non-polar lipid and glycolipid in the bound lipid content.

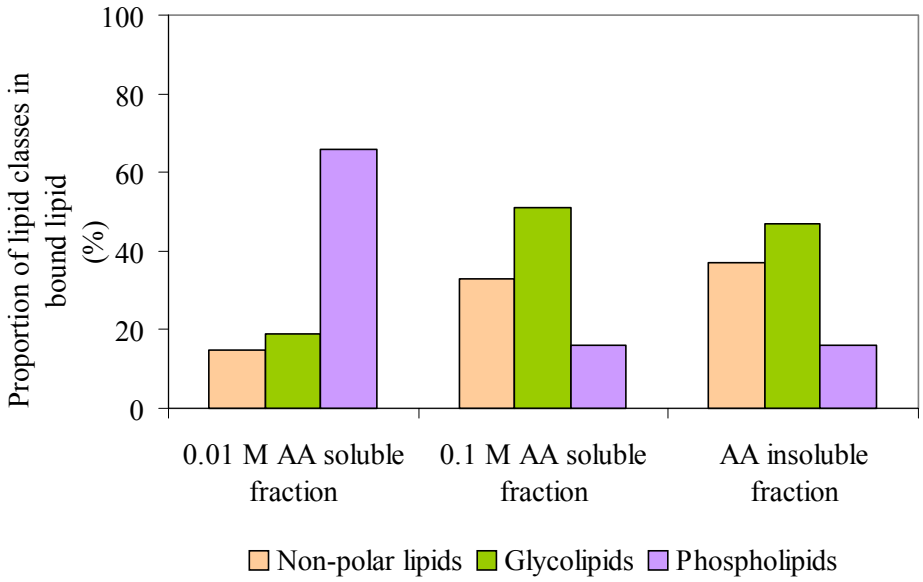


Figure 10.4 Pattern of lipid class distribution in bound lipid extract from the sequential acetic acid fractions
Note AA represents acetic acid

10.4.2 Pattern of lipid classes of free and bound lipid distributed in the sequential acetic acid fractions

The differences and similarities of lipid distribution in the three sequential fractions were further evaluated with a comparison of the level of single lipid classes. For the free lipid, the 0.01 M acetic acid soluble and the acetic acid insoluble fraction had similar patterns of lipid distribution, as there were no significant differences in the amounts of non-polar lipid and the proportions of TAG, DAG and MAG were similar (Table 10.6 and Figure 10.5). On another hand, the 0.1 M acetic acid soluble fraction contained a significantly higher amount of non-polar lipid ($P<0.01$) and a different distribution of TAG, DAG and MAG compared to the 0.01 M acetic acid soluble and insoluble fractions. All the three fractions were relatively high in TAG and low in DAG and MAG; however, the proportion of DAG and MAG was higher in the 0.1 M acetic acid soluble fraction (17 and 19%, respectively) than in the 0.01 M acetic acid soluble and acetic acid insoluble fractions (3–6 and 9–10%, respectively).

Table 10.6 Distribution of lipid classes as free and bound lipid following sequential fractionation

	Sequential acetic acid fractionation		
	0.01M acetic acid soluble fraction	**0.1M acetic acid soluble fraction**	**Insoluble fraction**
Free lipids			
Non-polar lipid	57 ± 10^{a}	607 ± 33^{b}	293 ± 131^{a}
TAG	(86)	(64)	(85)
DAG	(10)	(19)	(9)
MAG	(3)	(17)	(6)
Glycolipids	0^{a}	311 ± 19^{b}	0^{a}
MGDG	0	(73)	0
DGDG	0	(27)	0
Phospholipids (PE, PS + PC)	0^{a}	76 ± 3^{b}	0^{a}
Bound lipids			
Non-polar lipids	82 ± 11^{a}	629 ± 57^{b}	415 ± 26^{c}
TAG	(59)	(68)	(73)
DAG	(5)	(7)	(7)
MAG	(36)	(25)	(20)
Glycolipids	103 ± 5^{a}	956 ± 43^{b}	530 ± 31^{c}
MGDG	(68)	(76)	(76)
DGDG	(33)	(24)	(24)
Phospholipids	367 ± 55^{a}	291 ± 2^{a}	182 ± 22^{b}
PE	(0.3)	(1)	(4)
PI	(0.7)	(3)	(3)
PS + PC	(12)	(84)	(84)
LPE	(34)	(0)	(0)
LPC	(53)	(11)	(9)

Notes: Values are in mg of lipid in 100 g of gluten
Data in parentheses are the proportion expressed as a percentage for the lipid class
Superscript letters are used to show statistically significant differences between means within the same row ($p<0.01$): values followed by the same letter are not significantly different

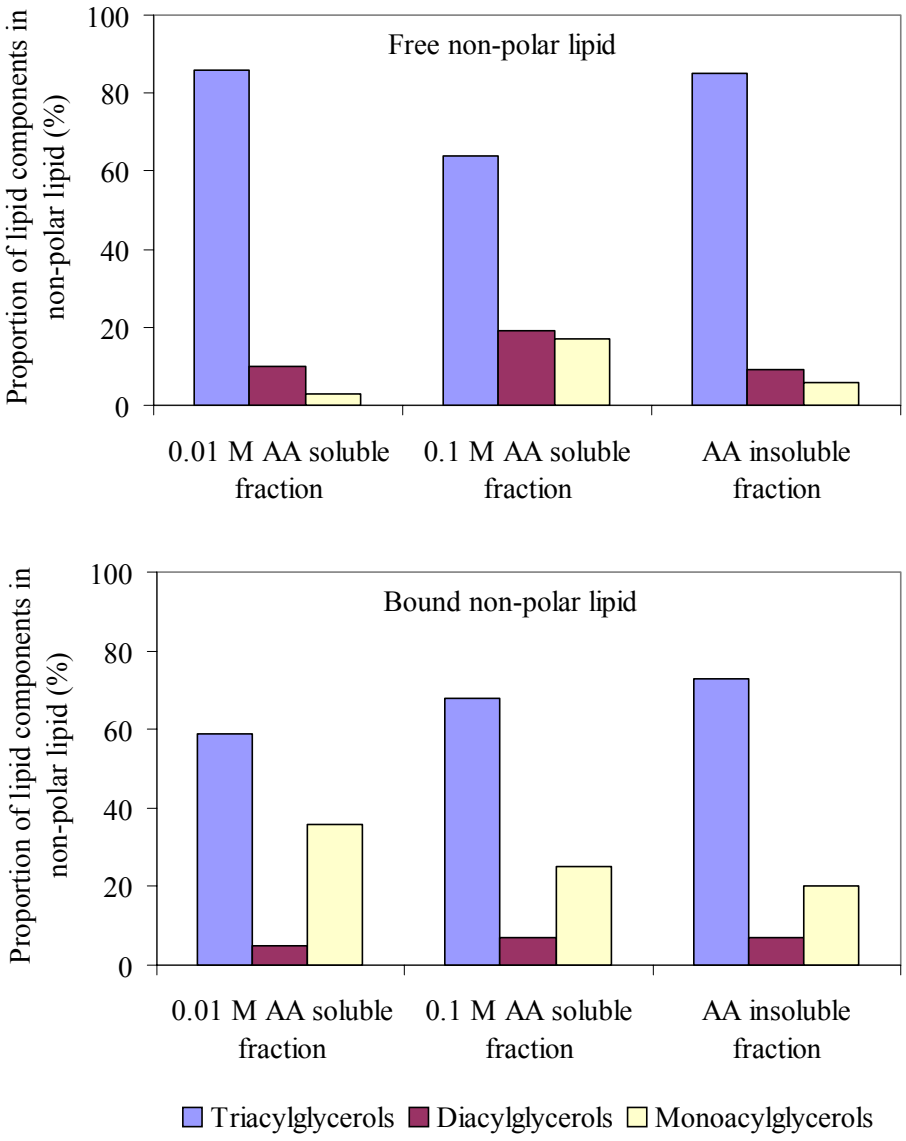


Figure 10.5 Pattern of lipid components distributed in the free and bound non-polar lipid of the sequential acetic acid fractions
Note AA represents acetic acid

A similar pattern of TAG, DAG and MAG distribution for the bound non-polar lipid was found in all three sequential fractions, in which TAG occurred as the highest proportion (59–73%), with MAG (20–36%) and DAG (5–7%) as the lowest (Table 10.6 and Figure 10.5). This pattern was also similar to the distribution of the free non-polar lipid in the 0.1 M acetic acid soluble fraction. Although the distribution patterns of bound non-polar lipids were similar within the three fractions, a relative increase was observed in the proportion of TAG and a decrease in MAG in the bound non-polar lipid

from the 0.01 M acetic acid soluble fraction to the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction. There was no change in the proportion of DAG in the bound non-polar lipid.

The distribution of glycolipids and phospholipids in the bound form in the 0.1 M acetic acid soluble fraction was similar to that of the acetic acid insoluble fraction but different from the 0.01 M acetic acid soluble fraction. This demonstrates that the ratio of MGDG to DGDG present as bound lipid was lower in the 0.01 M acetic acid soluble fraction (2:1) than in the 0.1 M acetic acid soluble fraction and the acetic acid insoluble fraction (3:1) (Table 10.6). It was observed that the ratio of MGDG and DGDG in the free lipid of the 0.1 M acetic acid soluble fraction was 3:1, the same as that in the bound lipid of the 0.1 M acetic acid soluble fraction and the acetic acid insoluble fraction. In relation to the phospholipids, the 0.1 M acetic acid soluble and acetic acid soluble fractions contained a high proportion of PS and PC (84%) but only minor amounts for the remainder of the phospholipids (3–9% for PE, PI, LPE and LPC). In contrast, the for 0.01 M there was a high proportion of LPE (34%) and LPC (53%) but low levels of PS and PC (12%), PI (0.7%) and (0.3%) (Table 10.6).

In summary, the results show that after the sequential fractionation of gluten with acetic acid, lipids were distributed at the highest level as both free and bound forms in the 0.1 M acetic acid soluble fraction. Non-polar lipids (TAG, DAG, MAG) were the major lipid components in free lipids isolated from all fractions, except for the 0.1 M acetic soluble fraction, in which glycolipids (MGDG and DGDG), together with small amount of phospholipids (PE, PS and PC) were found as part of the free lipids. Analyses of the bound lipids showed that the 0.01 M acetic acid soluble fraction contained a relatively high proportion of phospholipid, while the 0.1 M acetic acid soluble and insoluble fractions contained relatively high amounts of glycolipids. Similar lipid distribution patterns were observed for the bound lipids found in the 0.1 M acetic acid soluble fraction and the insoluble fractions. Therefore, the different level of these lipids between these two fractions can be attributed to differences in protein recovery.

10.5 The relationship between lipid and protein distribution in the three sequential acetic acid fraction

10.5.1 The pattern of free-lipid distribution in relation to the protein distribution in the three sequential acetic acid fractions

The pattern of free lipid distribution in the three sequential fractions was found not to directly relate with the content or pattern of protein in these fractions. The result showed that the 0.01 M acetic acid soluble fraction and the acetic acid insoluble fraction had similar patterns of free-lipid distribution but differences in the pattern of protein composition (Figures 10.3 and 10.6). Non-polar lipids are the major components of the free lipids in these fractions. In the protein distribution, gliadins predominate in the 0.01 M acetic acid soluble fraction whilst glutenins are in the majority for the acetic acid insoluble fraction. On the other hand, the 0.1 M acetic acid soluble fraction and the acetic acid insoluble fraction have the same protein pattern but a difference in the pattern of free lipid distribution (Figures 10.3 and 10.6). Both 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction contain a high proportion of glutenins in their protein content; however, there is a difference in the free lipid distribution. The free lipid of the 0.1 M acetic acid soluble fraction consists of non-polar lipid, glycolipid and phospholipid, while the acetic acid insoluble fraction contains only non-polar lipid in its free lipid extract. The lack of an obvious relationship between the free lipid pattern and the protein pattern in the three sequential fractions suggests that the distribution of free lipid could be related to the effect of acetic acid on gluten structure or lipid solubility rather than the association of lipid and gluten protein.

10.5.2 The pattern of bound lipid distribution in relation to the protein distribution in the three sequential acetic acid fractions

The pattern of distribution of the bound lipid in the three sequential fractions was found to directly correspond to the protein pattern or protein content in these fractions (Figure 10.6). The 0.01 M acetic acid soluble fraction contains a unique lipid pattern with the high proportion of phospholipid and a distinctive protein pattern with a low proportion of glutenins and high proportion of gliadins (Figures 10.3 and 10.6). On the other hand, the 0.1 M acetic acid soluble fraction and the acetic acid insoluble fraction have similar lipid distribution and protein pattern (Figures 10.3 and 10.6), illustrated by the high

amount of glycolipids in the bound lipid and the dominant proportion of glutenin in their protein components. Furthermore, the amounts of these lipids were reduced from the 0.1 M acetic acid soluble fraction to the acetic acid insoluble fraction proportionally to the reduction of the protein content. The correlation of protein and bound lipid distributions suggested the interactions of specific proteins and lipids when they exclusively come together in the gluten fraction.

The interactions of gluten protein and lipid classes were further elucidated through an evaluation of the amount of bound lipid per a gram of protein in the sequential acetic acid fractions. Bound non-polar lipids (TAG, DAG and MAG) and bound glycolipids (MGDG and DGDG) were more closely associated with proteins in the 0.1 M acetic acid soluble and acetic acid insoluble fractions than in the 0.01 M acetic acid soluble fraction, illustrated by the higher amount of bound lipid per a gram of protein (Figure 10.4). In phospholipids, high amounts of bound LPE and LPC per gram of protein were found in the 0.01 M acetic acid soluble fraction while high amounts of PS + PC per gram of protein occurred in the 0.1 M acetic acid soluble and acetic acid insoluble fractions, demonstrating the preferential interaction of LPE and LPC with protein in the 0.01 M acetic acid soluble fraction, whilst PS + PC were probably associated with protein in the 0.1 M acetic acid soluble and insoluble fractions. It was also noted that although the amounts of bound lipid in 100 g of gluten were higher in the 0.1 M acetic acid soluble fraction than in the acetic acid insoluble fraction (Table 10.6), the amounts of bound lipid per a gram of protein were similar for these two fractions (Figure 10.7). This was interpreted as indicating that the extent of association between protein and lipid in these fractions was quite similar.

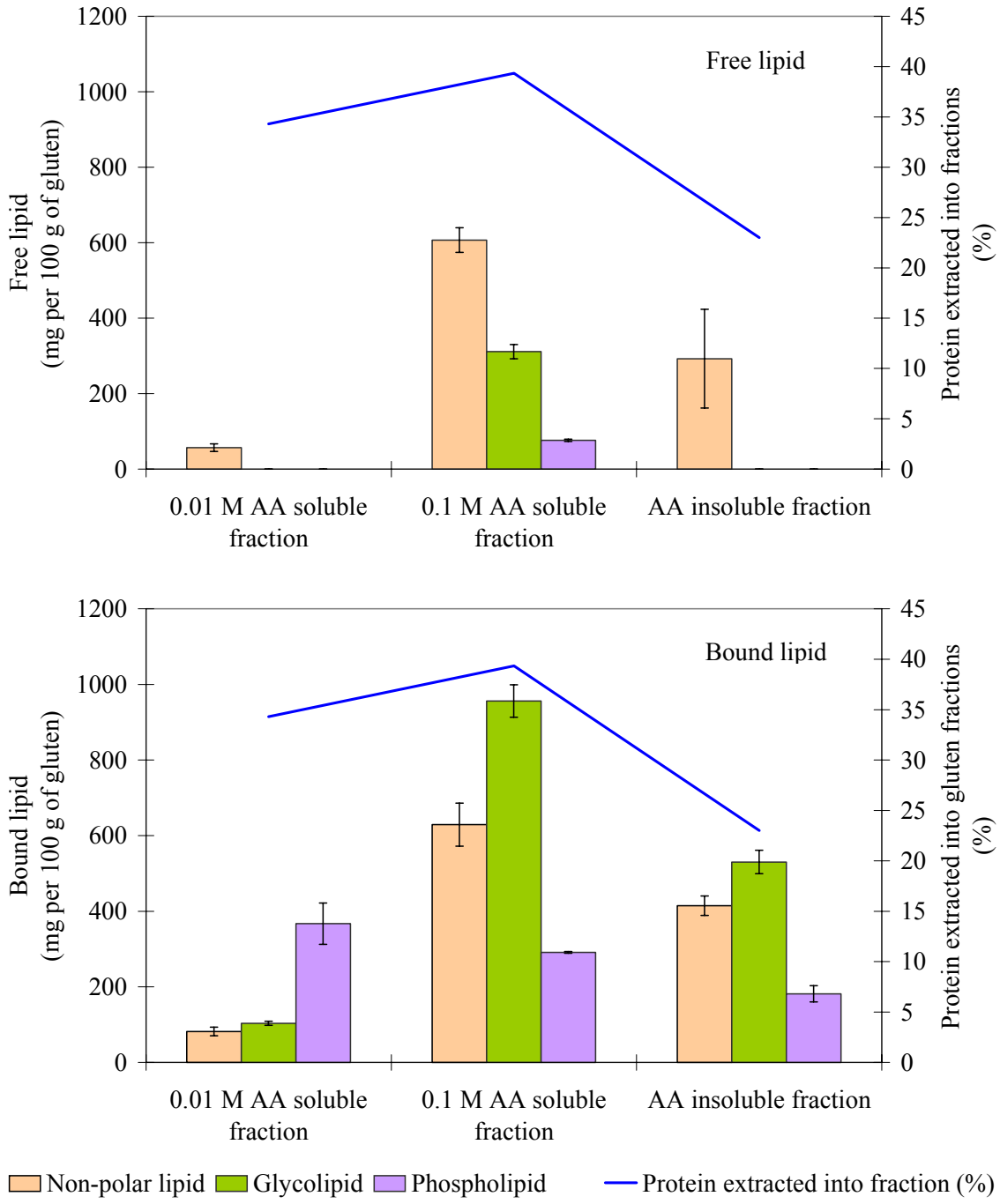


Figure 10.6 Relationship of free and bound lipid distributions and level of protein recovery in the sequential fractions

Note AA represents acetic acid

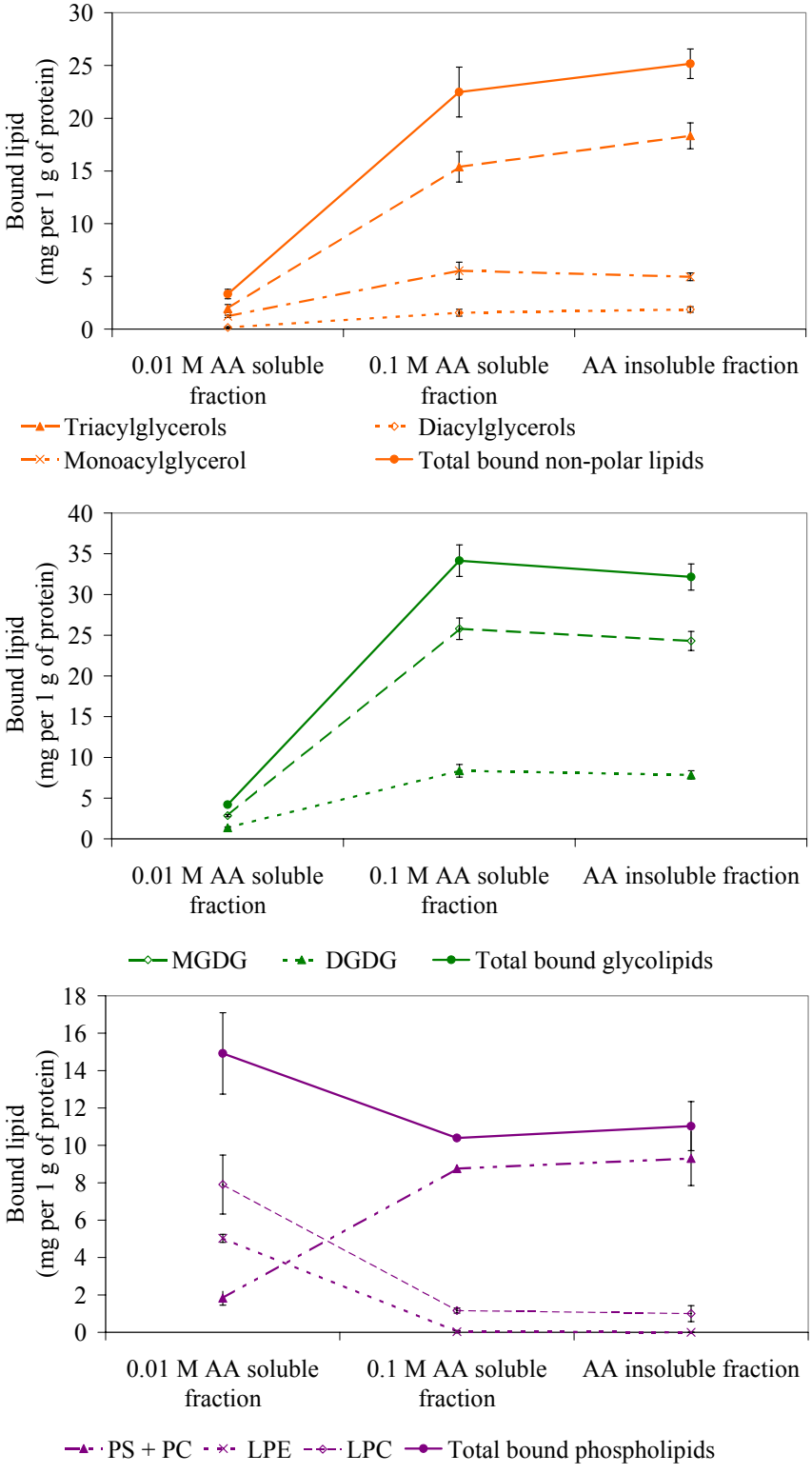


Figure 10.7 The distribution of bound lipids per g of protein in the sequential fractions

10.6 General discussion and summary

The lack of relationship between the patterns of the free lipids and the proteins is interpreted as indicating that the increasing amount of free lipids, especially non-polar lipids in gluten after acetic acid fractionation could be attributed to the effect of acetic acid on the structure of gluten protein rather than the interaction of protein and lipid (Section 10.5.1). Gluten contains a large number of hydrogen bonds and mixing gluten with acetic acid solution could cause disruption of the hydrogen bonds between glutenin and gliadin, facilitating the re-orientation of gluten protein (Bushuk, 1998; Wrigley et al., 2006). The re-arrangement of gluten structure could permit some lipids, that are usually retained within the gluten matrix due to physical entrapment (Marion et al., 1987), to be effectively released from entrapment as they encounter solvent molecules so that they can be extracted as free lipid. At the first stage of the fractionation with 0.01 M acetic acid, most of the free lipid remains in the pellet (Chapter 9) whereas at the second fractionation step, when 0.1 M acetic acid is used, most of the free lipids appear in the soluble fraction. The effective separation of particular gluten protein fractions, as a result of the use of acetic acid solutions having different concentrations, appears to have an affect on the distribution of free lipid in the resultant fractions.

Based upon the electrophoretic patterns for the proteins from the sequential acetic acid fractionation of gluten, the two concentrations of acetic acid selectively separated monomeric and polymeric proteins present in the gluten. Although this fractionation did not produce fractions having 100% purity of monomeric or polymeric proteins, a low concentration of acetic acid (0.01 M) is effective for extraction of monomeric protein while a higher concentration of acetic acid (0.1 M) was more effective for the solubilisation of polymeric protein. It has previously been suggested that the separation of monomeric and polymeric proteins primarily depends on the concentration of acetic acid as well as the ratio of gluten and solvent (Berot et al., 1994). In a gluten matrix, α-, β-, γ- and ω- gliadins are not able to form the intra-chain linkages through disulfide bonds. Hence their structure and contribution to the gluten matrix is stabilised by hydrogen bonds. In contrast, disulfide bonds, hydrogen bonds and hydrophobic interactions contribute directly to the formation of inter- and intra-chain bonds between HMW-GS, LMW-GS and other proteins (Shewry et al., 1992; Shewry and Tatham,

1997; Bushuk, 1998; Wrigley et al., 2006). This would explain why monomeric proteins could be dissociated from the gluten matrix at the low acetic acid concentration (0.01 M) while polymeric proteins required a higher level of acetic acid (0.1 M) in order to increase their solubility (or extractability).

After monomeric proteins are dissociated from the gluten matrix at the lower concentration of acetic acid (0.01 M), free lipid components, primarily non-polar lipids preferentially remain in the glutenin-rich fraction (pellet) rather than in the gliadin-rich fraction (0.01 M acetic acid soluble fraction). This is consistent with non-polar lipids being entrapped between gliadin and glutenin in the gluten network (Marion et al., 1987). The non-polar lipids might remain on the surface of the glutenin through the hydrophobic interactions after gliadins are removed. As a result, they can subsequently be extracted with a non-polar solvent (petroleum ether). It was also observed that non-polar lipids were distributed evenly between free and bound lipid in each protein fraction (Figure 10.6), indicating that non-polar lipid could be associated with gluten protein in a non-specific way. This finding appears to confirm the report from Marion et al. (1987) who specified that all gluten lipids exist in lipid vesicles entrapped within the gluten network. However, in contrast to these previous findings, the current study demonstrates that the entrapment of lipid in gluten occurs only for the non-polar lipids, whilst polar lipids interact with proteins through specific bonding mechanisms, which will be discussed in a later paragraph of this Chapter.

The polymeric proteins were affected by a high concentration of acetic acid (0.1 M) with the result that they became more soluble; this corresponded with a small amount of glycolipid being found in the free lipid extract. The dissociation of polymeric protein from the gluten matrix coinciding with the appearance of free glycolipid could suggest the entrapment of glycolipids within polymeric proteins. However, the distribution of free and bound glycolipids in the sequential acetic acid fractions could be interpreted in another way. Free glycolipids are only found in the 0.1 M acetic acid soluble fraction (Figure 10.6). Of the total glycolipid, a large amount occurs as bound lipid in the 0.1 M acetic acid soluble and acetic acid insoluble fractions whilst only a small amount is present in the free lipid extract of the 0.1 M acetic acid soluble fraction. Glycolipids can interact with protein through hydrophobic interactions as well as via hydrogen bonds (Hoseney et al., 1970a). As acetic acid is able to disrupt hydrogen bonds or change the

surface charge of proteins, the hydrogen bond between glycolipids and glutenin might be weakened, and so the glycolipids can then be extracted with petroleum ether. This explanation was further supported by the absence of glycolipids in the free lipid extract of the acetic acid insoluble fraction, the protein of which shows less effect of acetic acid on the surface charge and hydrogen bonds. The evidence supports the occurrence of interactions between glycolipids and glutenins, rather than the entrapment of these lipids within the structure of the gluten matrix.

The correspondence between the protein pattern and the bound lipid distribution within the sequential acetic acid fractions indicates the association of protein and lipid related to the co-existence of bound lipid and protein in gluten fractions (Section 10.5.2). The relatively high levels of bound lipid, found in the 0.1 M acetic acid soluble fraction, indicate that lipid was preferentially associated with those gluten proteins solubilised at high acetic acid concentration, and this is similar to observations made in previous reports (Frazier et al., 1981; Frazier, 1983). The interactions of protein and lipid in the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction are similar as they have the same pattern of protein and lipid distribution and no significant differences in the amounts of bound lipid per gram of protein although the protein contents in these fractions are different (Figures 10.6 and 10.7). As much of the protein in the acetic acid insoluble fraction is polymeric glutenin (Figure 10.2), the association of protein with bound lipids in the 0.1 M acetic acid soluble and acetic acid insoluble fractions primarily involves HMW-GS and LMW-GS.

Non-polar lipid and glycolipids are the major bound lipids in the 0.1 M acetic acid soluble and acetic acid insoluble fractions containing a high proportion of glutenin. This indicates the association of these lipids with glutenin, which is consistent with previous findings (Chung and Tsen, 1975a, 1975c; Frazier et al., 1981; Bushuk, 1985; Chung, 1986). However, non-polar lipids might not specifically interact with glutenin, but rather they might be locked within the gluten matrix formed between gliadins and glutenins. Glycolipids interact with glutenins that were either soluble or insoluble in a dilute acetic acid solution, as demonstrated by the presence of similar amounts of bound glycolipid components per gram of protein (Figure 10.7). In addition, the similar ratio between MGDG and DGDG was found in both 0.1 M acetic acid soluble and acetic acid insoluble fractions (Figure 10.5 and Table 10.6). Furthermore, the ratio of MGDG and

DGDG in the free lipid extract of the 0.1 M acetic acid soluble fraction is the same as in the bound-lipid extract. This supports the possibility that acetic acid has an effect on polymeric glutenin, weakening the association of glutenins and glycolipids, so that some of them can be extracted with petroleum ether.

As the high proportion of phospholipids occurred in the 0.01 M acetic acid soluble fraction containing a high level of gliadins, this indicated that phospholipids preferentially associated with gliadins. Furthermore, it was observed that the levels of phospholipid decreased from the 0.01 M acetic acid soluble fraction to the 0.1 M acetic acid soluble and insoluble fractions, while the densities of protein bands at MW of approximately 12, 16, 26.8 and 42 kDa also decreased (Figures 10.3 and 10.6). These findings suggest that these proteins preferentially associate with phospholipids. Proteins with the similar MW of 12, 16, 26.8 and 42 kDa, have been found in the S protein fraction which was able to associate with polar lipids (Zawistowska et al., 1986). Puroindolines, having 12.8 kDa MW, have been found to have a high affinity for phospholipids and glycolipids (Dubreil et al., 1997; Kooijman et al., 1997). CM proteins containing a MW of 12 to 13 kDa are known to be lipoproteins accumulating with the lipid extract on the interface (Meredith et al., 1960; Carr et al., 1992). This provides evidence that the 0.01 M acetic acid soluble fraction contains a number of proteins able to associate with phospholipids. The amounts of bound LPC and LPE per gram of protein were high in the 0.01 M acetic acid soluble fraction, indicating that proteins at MW of approximately 12, 16, 26.8 and 42 kDa might preferably interact with LPC and LPE. High amounts of bound PC and PS per gram of protein occur in the 0.1 M acetic acid soluble fraction and in the acetic acid insoluble fraction, indicating the interaction of PC and PS with proteins in these fractions. However, further characterisation of proteins in the sequential acetic acid fractions is required in order to explain the interaction of protein and lipid in gluten, based upon the distribution of protein and lipid components in the gluten fractions.

Chapter 11

Results and discussion: Further characterisation of protein and lipid in the sequential acetic acid fractions

11.1 Introduction

Gluten proteins are predominantly glutenins and gliadins, the classification of which has been established based on solubility (Osborne, 1924), electrophoretic mobility (Shewry, 2003) and amino acid sequences (Tatham, 1995). The classification and structure of these proteins have been reviewed and discussed in Section 4.2. Glutenins primarily consist of two subunits: HMW-GS and LMW-GS, whilst gliadins contain different types which are designated α, β, γ and ω. LMW-GS and α, β, γ-gliadins have a similar mass and belong to the S-rich prolamins as they are all relatively high in cysteine. ω-Gliadins lack cysteine residues and are therefore classified as S-poor prolamins.

Glutenin and gliadins have been reported to associate with lipids during dough mixing or gluten preparation (Frazier, 1983; Chung, 1986; Carr et al., 1992). In addition, it has been found that puroindolines and lipid transfer protein, which are classed as lipid binding proteins in flour, are able to interact with lipids, particularly polar lipids (Marion et al., 2003). In the previous chapter (Chapter 10), the distributions of lipid and protein in the three sequential acetic acid fractions indicated the preferential association of phospholipids with LMW-GS and gliadins as well as of glycolipids with glutenin. Most of the proteins in the acetic acid insoluble fraction are glutenins, which are known to have the ability to form a large protein network of HMW-GS and LMW-GS linked through disulfide bonds (Shewry and Tatham, 1997).

Within the broad objective of extending the understanding of the interactions between protein and lipid components in gluten, the aim of this phase of the investigation has been firstly to further identify which specific protein or proteins are associated with lipid and, secondly, to assess the impact of disulfide bonds on the interactions of lipid and protein. The approach has been to further analyse and characterise proteins,

particularly to determine the presence of lipoproteins in the three sequential acetic acid fractions, and also to investigate the effect of reducing agent on interactions of lipid and glutenin existing in the acetic acid insoluble fraction.

11.2 Amino acid composition

Apart from differences in the MW and electrophoretic mobilities, glutenin and gliadin can be differentiated on the basis of amino acid composition. It has been reported that all gliadin components are rich in glutamine and proline but low in basic amino acid especially in lysine (Tatham, 1995; Lasztity, 1996). On the other hand, glutenins are relatively lower in glutamine and higher in lysine and asparagine, compared to gliadin (Lasztity, 1996). In the previous chapter (Chapter 10), the SE-HPLC and SDS-PAGE results have demonstrated that the sequential acetic acid fractionation is able to selectively separate gluten proteins into gliadin- and glutenin-rich fractions. Hence, it is expected that the amino acid composition of the three sequential acetic acid fractions will be different.

The amino acid compositions of the three sequential fractions (0.01 M acetic acid soluble, 0.1 M acetic acid soluble fraction and the insoluble fraction) are presented in Table 11.1. The results are compared with literature values for gliadin, glutenin and pH glutenin fractions (Lasztity, 1996). It is noted that these were obtained for gliadin and glutenin prepared by gel filtration chromatography and the proteins designated as pH glutenin were prepared by a precipitation of the AUC (0.1 M acetic acid, 3 M urea and 0.01 M cetyltrimethylammonium bromide) extract of flour protein at pH 6.4 (Wasik and Bushuk, 1974).

The three acetic acid fractions show patterns of amino acid composition that are similar to literature values reported for gliadin, glutenin and pH glutenin. Firstly, the amino acid composition of the 0.01 M acetic acid soluble fraction is similar to gliadin, as it is relatively high in glutamine and proline and low in basic amino acids including lysine, arginine and histidine compared to other fractions. This indicates that the majority of proteins in the 0.01 M acetic acid soluble fraction are gliadins.

The 0.1 M acetic acid soluble and acetic acid insoluble fractions have relatively low contents of glutamic acid and proline and are higher in basic amino acids, especially lysine, arginine and aspartic acid. Based upon the composition, the predominant proteins in the 0.1 M acetic acid soluble and acetic acid insoluble fractions are glutenin although the former contain a certain proportion of gliadin.

Table 11.1 Amino acid composition of the sequential acetic acid fractions in comparison with literature values

	Sequential acetic acid fractionation			Literature values (Lasztity, 1996)		
	0.01 M acetic acid soluble	**0.1 M acetic acid soluble**	**Acetic acid insoluble**	**Gliadin**	**Glutenin**	**pH glutenin**
Lys	0.9	1.7	3.0	0.6	1.5	4.3
His	1.6	1.7	2.0	1.8	1.5	2.0
Arg	2.4	2.8	3.9	1.8	2.4	4.5
Asp	2.9	3.2	4.6	2.4	2.7	6.6
Thr	2.5	2.8	3.6	2.2	3.1	4.3
Ser	5.9	6.2	6.3	4.6	6.0	6.8
Glu (acid)	37.3	35.5	29.1	38.0	33.1	23.0
Pro	15.4	13.7	11.2	17.7	13.6	8.7
Gly	5.2	7.5	8.1	3.0	9.3	8.4
Ala	3.4	3.8	5.1	3.0	4.1	6.4
Val	4.4	4.1	4.9	5.2	4.9	5.3
Met	1.2	1.2	1.4	1.4	1.4	0.8
Iso	3.9	3.4	3.8	4.4	3.3	4.2
Leu	6.8	6.4	7.0	7.4	6.8	8.1
Tyr	2.3	2.6	2.4	1.9	3.0	2.7
Phe	3.9	3.6	3.5	4.6	3.2	3.9

Notes 1 The fractions gliadin, glutenin and pH glutenin for which literature values are presented are further described in the text (section 11.2, paragraph 1)
2 The original analyses for the sequential acetic acid fractions were carried out by the Australian Proteome Analysis Facility and the data are presented in Appendix 1?
3 All values of amino acids expressed as mole percentage

In further comparing the 0.1 M acetic acid soluble and acetic acid insoluble fractions with the literature figures for the amino acids (Table 11.1), the 0.1 M acetic acid soluble fraction is closer in composition to general glutenin. On the other hand, the acetic acid insoluble fraction has a pattern similar to that of the fraction designated as the pH glutenin. Both the acetic acid insoluble fraction and the pH glutenin had high contents of lysine, histidine, arginine, asparagine, threonine, serine, alanine and valine and the lowest levels of glutamic acid and proline.

11.3 Characterisation of protein in the sequential acetic acid fractions using two-dimensional electrophoresis

Glutenins and gliadins are comprised of many subunits, which vary in MW and pI. SE-HPLC and SDS-PAGE are commonly used for characterising gluten protein, and both primarily separate on a basis of differences in MW. Recently, 2DE has provided extremely high resolution in the separation of polypeptides reflecting differences in both net-charge and size. This technique has been used effectively for the characterisation of proteins from wheat grains (Skylas et al., 2000; Islam et al., 2003; Skylas et al., 2005). Therefore, 2DE has been selected for further characterising the three sequential acetic acid fractions in order to compare the distribution of protein in these fractions.

Proteins of the three sequential fractions were separated by 2DE using a non-linear strip pH 3.0–10.0 for the IEF step and Bis-Tris gradient gel (4–12%) for the SDS-PAGE step. The gel images of proteins from the three fractions are displayed in Figure 11.1. The general patterns of proteins on these gels were consistent with previous reports (Islam et al., 2003; Skylas et al., 2005), however, some minor differences were observed in the number of protein spots. This can probably be attributed to the difference of protein between wheat grain and wheat gluten. The previous studies (Islam et al., 2003; Skylas et al., 2005) characterised proteins from wheat grains whilst this study focused on those from wheat gluten. In addition, the differences of the spots on the 2DE image may also be due to variations in the specific gels employed in the two studies.

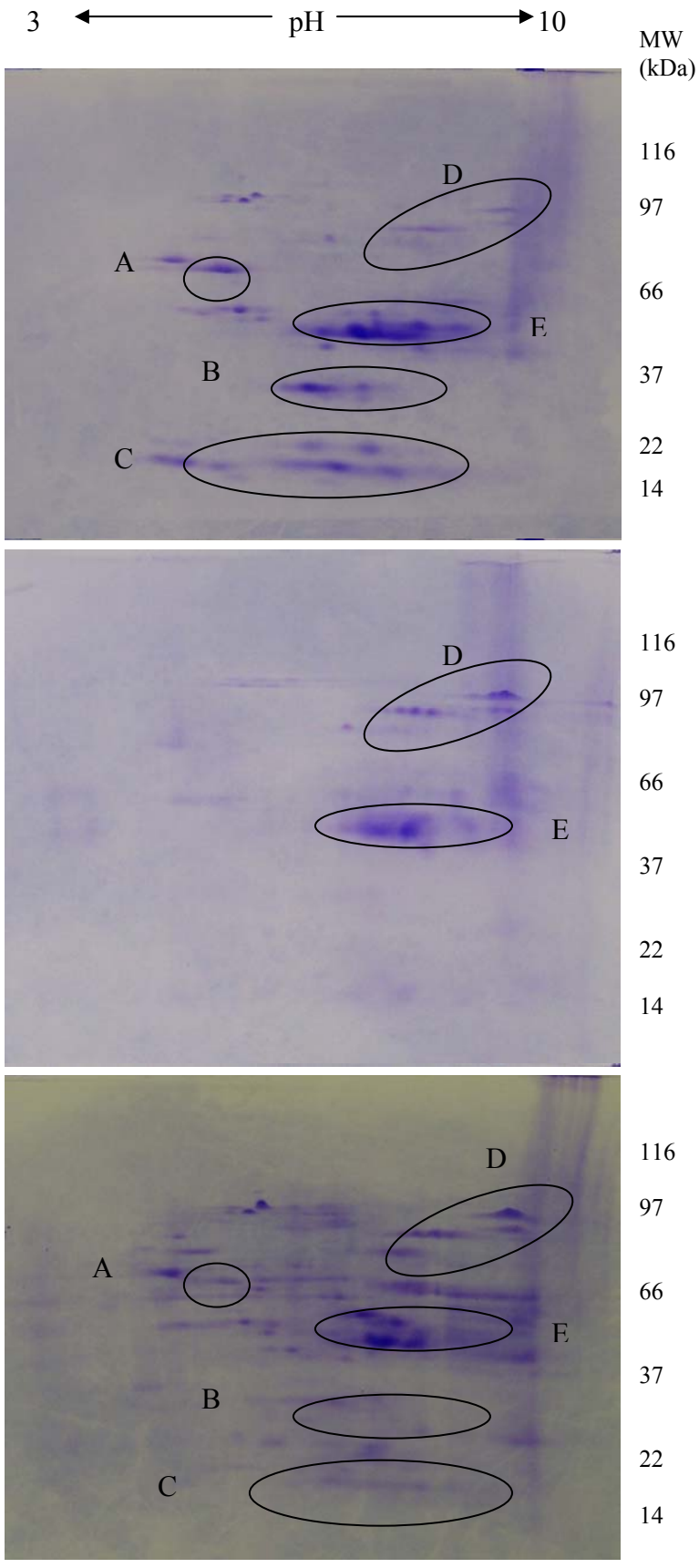


Figure 11.1 Two dimensional electrophoresis (IEF and SDS-PAGE) of the sequential acetic acid fractions

In presenting the 2DE patterns (Figure 11.1), specific regions have been circled and labelled as A, B, C, D and E. These represent the proteins which vary considerably between the three sequential fractions and which are the focus for the comparison of the proteins in the three fractions. The likely identity of the proteins in the regions and their presence in the three fractions are summarised in Table 11.2.

On the 2DE image of 0.01 M acetic acid soluble fraction, a number of protein spots appear in the regions of apparent MW from 80 to 14 kDa. These regions of MW are designated to LMW-GS, gliadins and non-storage proteins. On another hand, the majority of protein spots on the 2DE gel of the 0.1 M acetic acid soluble fraction occur in the region of MW above 37 kDa, which have been known as HMW-GS, LMW-GS and gliadins. The 2DE image of acetic acid insoluble fraction demonstrated a similar pattern to that of the 0.1 M acetic acid soluble fraction. This is consistent with the result of the one-dimensional electrophoresis on SDS-PAGE gel showing a similar pattern of protein bands between these two fractions (Figures 10.2 and 10.3). It was noted that the density of some protein spots on the 2DE gel of the 0.1 M acetic acid soluble fraction were too soft to observe. This could be due to the relatively low protein concentration that can effectively enter into the gel.

In a comparison of 2DE images of protein, there were similarities observed for the three fractions with the predominant appearance of protein spots in the circle E (Figure 11.1) and these are most likely to be LMW-GS and gliadins (Table 11.2). The comparison showed that the distribution of protein spots on the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction were quite similar but different to that of the 0.01 M acetic acid soluble fraction. Protein spots in the circle A, B and C, which are likely gliadins, LMW-GS and non-storage proteins occur dominantly on the gel of the 0.01 M acetic acid soluble fraction, whereas these spot were not detected on the 2DE of the acetic acid insoluble fraction or only faintly present on that of the 0.1 M acetic acid soluble fraction. The protein spots in the circle B and C in the Figure 11.1 appear at the same region of some protein on the 2DE gel image of amphiphilic protein of wheat extracted with TX114 (Amiour et al., 2002). This indicates the possible occurrence of amphiphilic protein in the 0.01 M acetic acid soluble fraction. These proteins in the three fractions are further investigated and discussed in Section 11.5.

Table 11.2 Distribution of protein spots on the 2DE images of the sequential acetic acid fractions

Circle	Protein	Image		
		1	2	3
		0.01 M acetic acid soluble	0.1 M acetic acid soluble	Acetic acid insoluble
A	Gliadins[1]	One dominant spot	None detected	Spots present
B	Possibly LMW-GS or gliadins	Spots present	None detected	None detected
C	Non-storage protein	Many strong spots	None detected	Faint spots
D	HMW-GS	Faint spots	Clear dominant spots	Clear dominant spots
E	LMW-GS and gliadins	Many strong spots	Many strong spots	Many strong spots

Note: 1 The dominant spot in region A of 2DE image of the 0.01 M acetic acid soluble fraction does not correspond to any of those seen in the acetic acid insoluble fraction

The protein spots in the circle D, known as the region of HMW glutenin (Islam et al., 2003), present predominantly on the gel of the 0.1 M acetic acid soluble and acetic acid insoluble fraction but fairly occur on that of the 0.01 M acetic acid soluble fraction. The difference of protein profile on the 2DE gel image within the three fractions indicates that glutenins are dominantly present in the 0.1 M acetic acid soluble and acetic acid insoluble fraction.

11.4 Analysis of protein in the sequential acetic acid fractions by RP-HPLC followed by SDS-PAGE

In order to further categorise the proteins in each of the three sequential fractions, the combination of RP-HPLC and SDS-PAGE has been investigated as an alternative approach to separation. In RP-HPLC, the most widely adopted HPLC method used for gluten protein analysis, the separation of protein is based on the differences of their surface hydrophobicities. On the other hand, with SDS-PAGE, proteins are separated on the basis of their size. The combination of these two methods is used to further

characterise the composition of proteins and to identify the differences of protein distribution in the three sequential fractions, based on both hydrophobicity and size.

Protein samples of the three sequential fractions were chromatographed using an HPLC system fitted with a C-18 column and in all cases, a series of 29 protein fractions were collected corresponding to the material eluted each minute from 25 to 54 min. Proteins of the collected fractions were then analysed using SDS-PAGE. The HPLC chromatograms and corresponding SDS-PAGE protein profiles of the 0.01 M acetic acid soluble fraction, 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction are shown in Figures 11.2, 11.3 and 11.4, respectively.

In each case, the general pattern of the elution profile of proteins from the reversed phase C-18 column is similar to those previously reported by (DuPont et al., 2005). The HMW-GS are eluted first and followed by a broad group of peaks which include LMW-GS and gliadins. The peaks eluted between 25 and 37 min correspond to the fractions 1−12 primarily containing HMW-GS because the protein bands of these fractions predominantly occur in the region of MW above 80 kDa on the SDS-PAGE gel (Figures 11.2, 11.3 and 11.4). LMW-GS and gliadins dominated in the peaks eluted from 37 to 54 min corresponding to fractions 13−29. This identification is based on the appearance of many protein bands in the region of MW from 31 to 55 kDa. In addition, some bands of non-storage protein with MW below 22 kDa were present although the pattern of these varied for the different acetic acid fractions.

Each of the three acetic acid fractions contained bands corresponding to HMW-GS, LMW-GS, gliadins and non-storage proteins although the proportions differed (Figures 11.2, 11.3 and 11.4). The 0.1 M acetic acid soluble and acetic acid insoluble fractions contained a high proportion of HMW-GS, demonstrated by the strong density of protein bands in the HMW-GS region. In order to more conveniently compare the protein bands present in the sequential acetic acid fractions, the results of proportion of peak area from the chromatograms have been summarised and are presented in Figure 11.5. This demonstrates that there were obvious differences between the acetic acid fractions. There is an increase of the proportion of the peak area in fractions 7, 9, 10 and 11 (Figure 11.5). This indicates that the relative amount of glutenin is higher in the 0.1 M acetic acid soluble and acetic acid insoluble fractions.

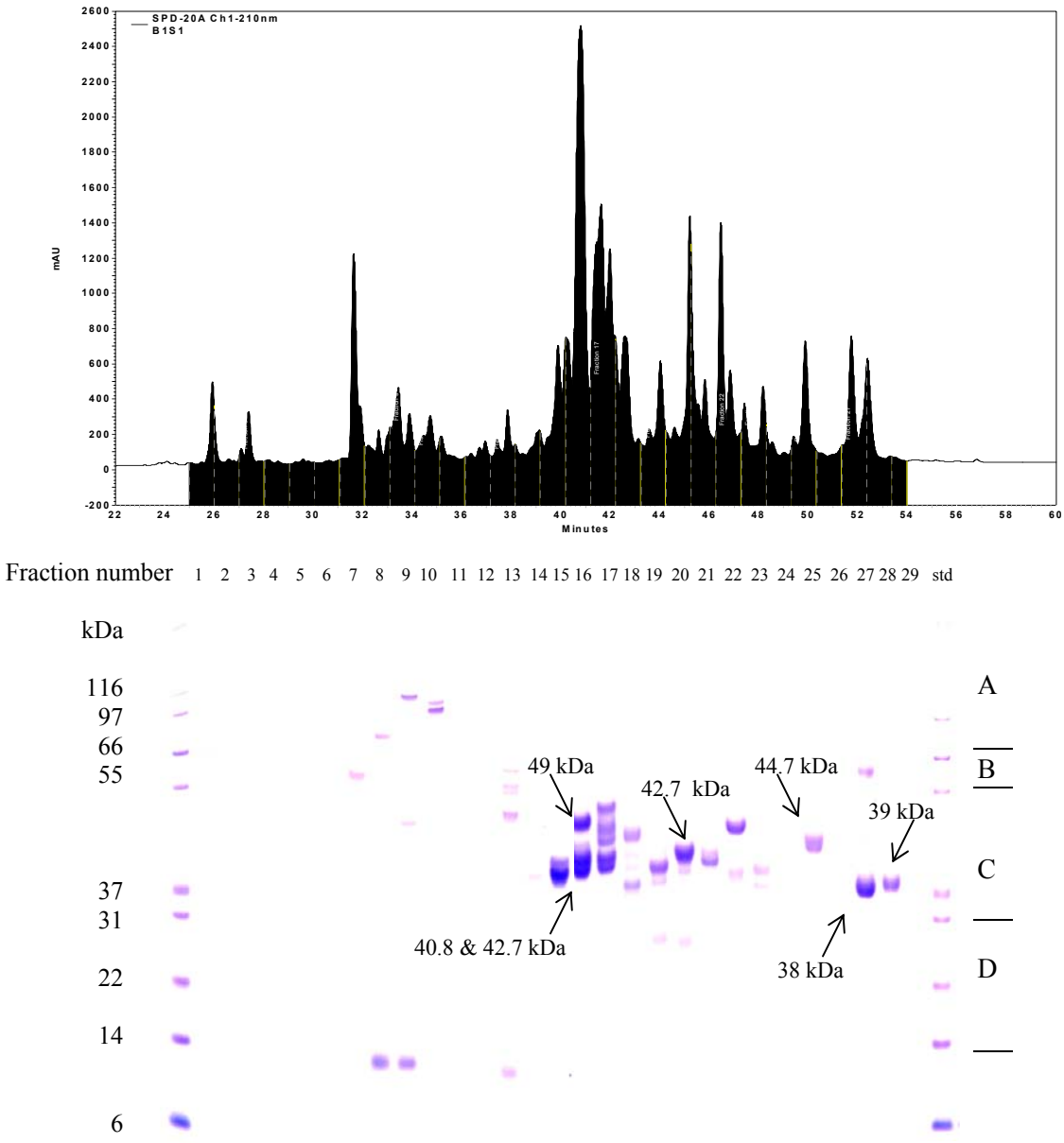
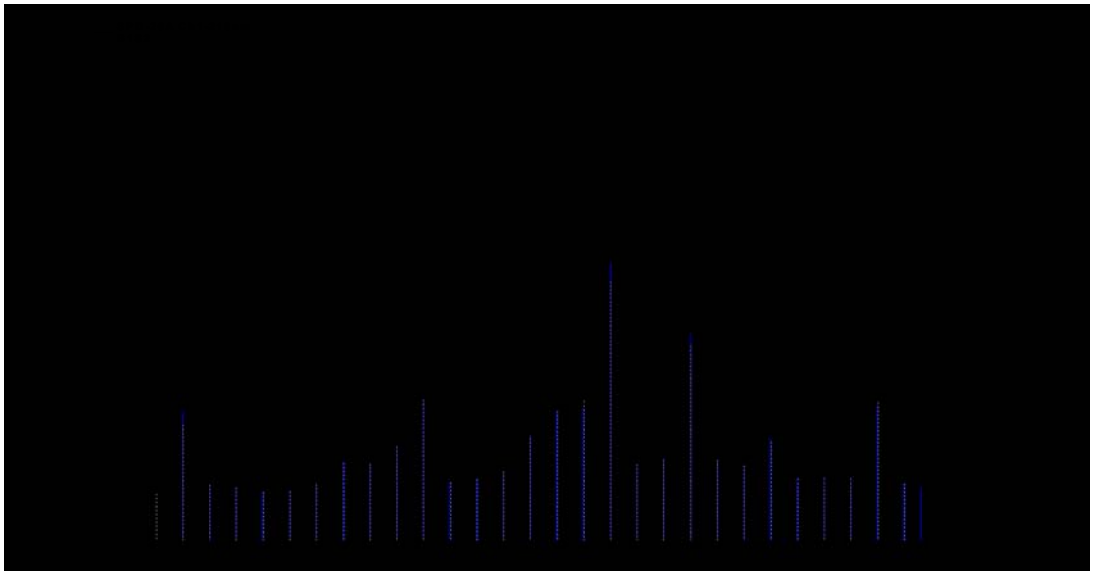


Figure 11.2 Characterising protein in the 0.01 M acetic acid soluble fraction using RP-HPLC and SDS-PAGE

Notes
A HMW-GS
B ω-gliadins
C, D LMW-GS and α,β,γ- gliadins



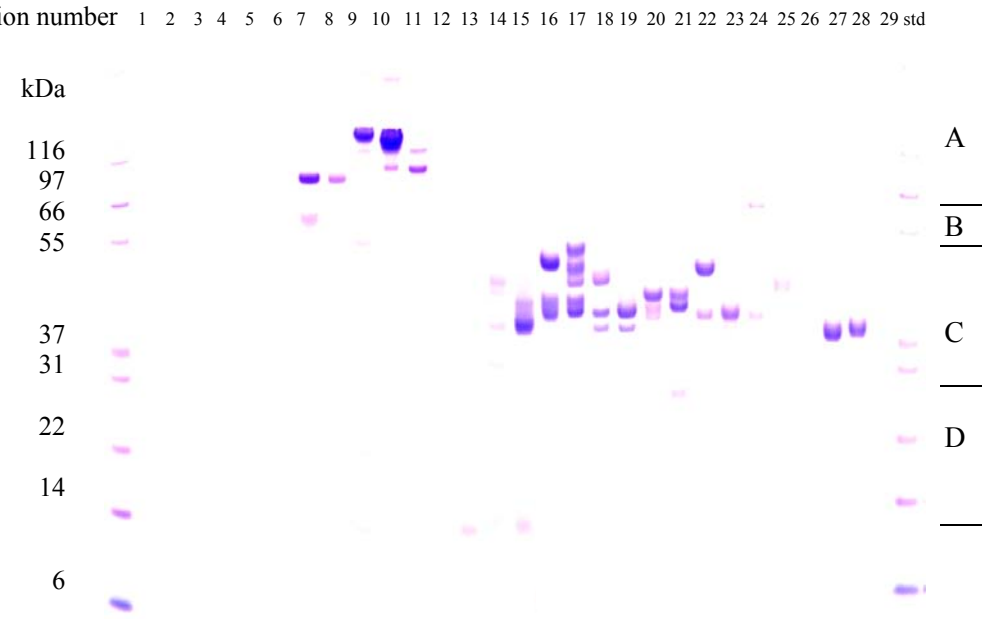
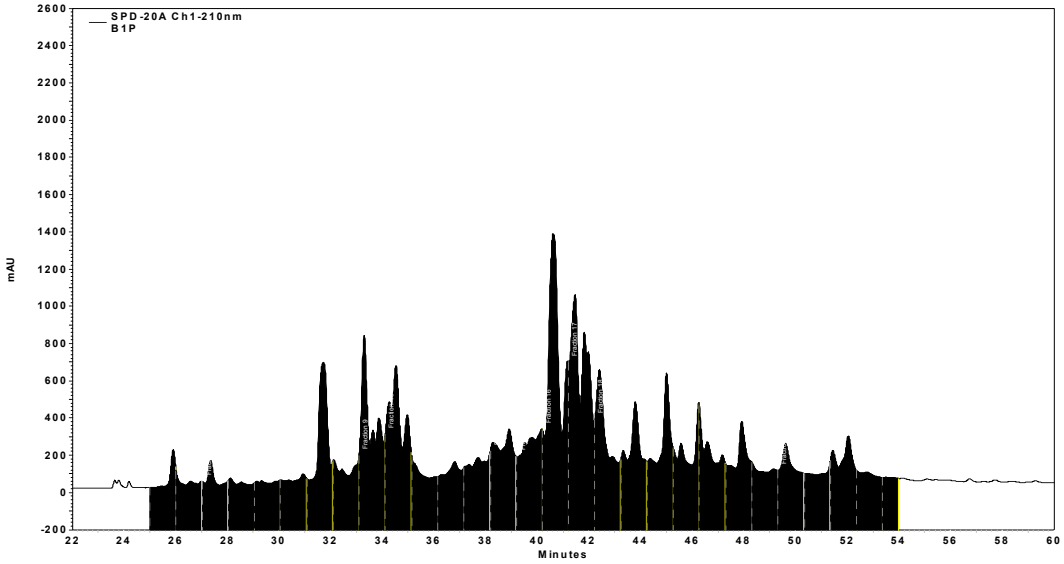


Figure 11.3 Characterising protein in the 0.1 M acetic acid soluble fraction using RP-HPLC and SDS-PAGE

Notes
A HMW-GS
B ω-gliadins
C, D LMW-GS and α,β,γ- gliadins



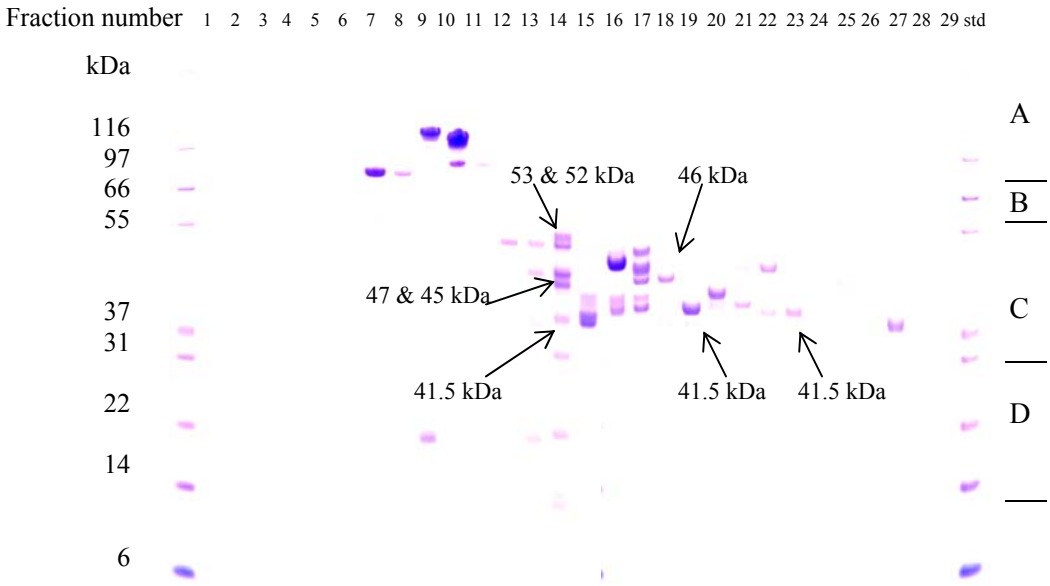


Figure 11.4 Characterising protein in the acetic acid insoluble fraction using RP-HPLC and SDS-PAGE

Notes A HMW-GS
B ω-gliadins
C, D LMW-GS and α,β,γ- gliadins

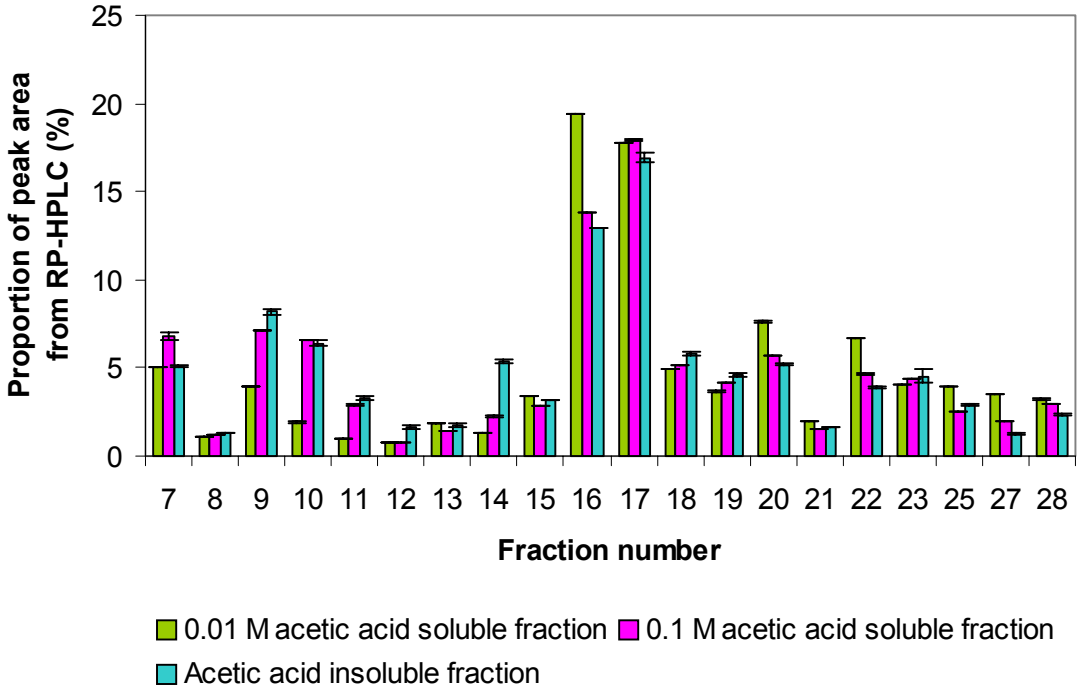


Figure 11.5 Relative amounts of protein in fractions eluted from RP-HPLC presented as a proportion of total peak area
Note: Results are presented as mean ± stdev for duplicate trials

On the HPLC chromatographs of protein in the three fractions, the pattern of protein peaks at 38–54 min, corresponding to fractions 14 to 29, was similar (Figures 11.2, 11.3 and 11.4); however, the percentage of area of those peaks varied (Figure 11.5). It was observed that the proportion of peak area was reduced in the fractions 16, 20, 22, 25, 27 and 28 and increased in the fractions 14, 18, 19 and 23 in the order of the 0.01 M acetic acid soluble fraction, the 0.1 M acetic acid soluble fraction and the insoluble acetic acid fraction (Figure 11.5). The SDS-PAGE results of the RP-HPLC fractions showed that the reduction of peak area proportion is related to LMW-GS or gliadin protein at the approximate MW of 38, 39, 40.8, 42.7, 44.7 and 49 kDa (Figure 11.2) and the increase of peak area proportion is involved in LMW-GS or gliadin proteins at approximate MW of 41.5, 45, 46.6, 52.4 and 53 kDa (Figure 11.4).

It is noted that a protein band having an approximate MW of 12 kDa was found with relatively strong intensity in fractions 8 and 9 of the 0.01 M acetic acid soluble fraction

(Figure 11.2) but could not be clearly seen in the other two acetic acid fractions (Figures 11.3 and 11.4). In addition, the proportion of the peak area in fraction 8 was relatively small (Figure 11.5). Further discussion on the distribution of the proteins in the three sequential fractions is presented in Section 11.5.

11.5 The occurrence of lipid-binding proteins in the sequential acetic acid fractions

Results from 2DE and the combination of RP-HPLC and SDS-PAGE demonstrated that the proteins at MW of 12 kDa, are probably amphiphilic and occur in reasonable amounts in the 0.01 M acetic acid soluble fraction (Sections 11.3 and 11.4). It has previously been reported that these proteins can be extracted from flour using non-ionic detergents and TX114 was found to be the most effective of these (Blochet et al., 1991). The lipid-binding proteins, PIN-a and PIN-b, have been purified and identified from the TX114 extract (Blochet et al., 1993; Kooijman et al., 1997). The presence of these proteins could be related to the distribution of lipid in the gluten fraction. Therefore, the objective of this part of the current study is firstly to investigate the TX114 extractability of protein in gluten and the sequential acetic acid fractions and secondly to identify the presence of PIN-a and PIN-b in these fractions. The results will be discussed in relation to the distribution of lipids in terms of the protein-lipid interactions in gluten.

11.5.1 Triton X114 extractability of proteins in flour, gluten and sequential acetic acid fractions

Flour, gluten and sequential acetic acid fraction samples were firstly fractionated into the pellets and supernatants using 4% TX114 (w/v). The supernatants were then partitioned to collect the TX114 extractable proteins. The extraction yields of these proteins are illustrated in Table 11.3. The pellet was termed as TX114 insoluble fraction. The SDS-PAGE protein patterns of TX114 extractable proteins and the TX114 insoluble fraction are presented in Figures 11.6 and 11.7, respectively.

The SDS-PAGE protein pattern of the TX114 extractable protein and the pellet from flour show that the distinctive protein band at MW of approximately 13 kDa is primarily present in the TX114 soluble proteins (Figure 11.6, lane a and b) whilst this

band is either absent or very faint in the TX114 insoluble fraction (Figure 11.7, lane a and b). This indicates that the conditions of the current TX114 procedure effectively extract the amphiphilic proteins from flour although the TX114 extract yield of flour in this study is lower (2.11−2.51%, w/w, total wheat flour protein) than the value reported previously (7.50%, w/w) (Blochet et al., 1991). The lower extraction yield could be due to the variation in experimental conditions as well as the different cultivars of wheat.

Table 11.3 The impact of TX114 on yields of protein from flour, gluten and sequential acetic acid fractions

	Extraction yield (%, w/w of total protein)
Flour (soft wheat, Rosella)	2.51 ± 0.10
Flour (hard wheat, Lang)	2.11 ± 0.60
Gluten (hard wheat, Lang)	1.45 ± 0.25
0.01 M acetic acid soluble fraction	2.72 ± 0.71
0.1 M acetic acid soluble fraction	1.74 ± 0.09
Acetic acid insoluble fraction	1.39 ± 0.25

The low TX114 extraction yield of gluten demonstrates that the proportion of lipid-binding proteins in gluten might be lower than that in flour (Table 11.3). This could be attributed to the association of PIN with starch granules (Bloch et al., 2001)that have been removed during the gluten preparation. In addition, results of SDS-PAGE showed that the protein band at MW of 13 kDa appeared at slightly higher intensity in the TX114 protein extract of gluten compared to that from flour (Figure 11.6, lanes a, b and c). The TX114 insoluble fraction of gluten still contained a considerable amount of the 13 kDa protein (Figure 11.7, lane c). These results indicate that this protein might be more strongly associated with other proteins or lipids in gluten than in flour, therefore it cannot be fully extracted under the same extraction conditions applied to flour. Apart from the 13 kDa protein, the TX114 extract also contained a significant amount of gliadin, primarily γ-gliadin and CM proteins as previously reported (Blochet et al., 1991). It was also observed that the protein of approximately 26 kDa was a strong band in the TX114 extract from gluten while it was not present in the TX114 extract from flour. This is similar in MW to a protein that has been isolated in the S proteins, which has been shown to have a high affinity with lipid (Zawistowska et al., 1985).

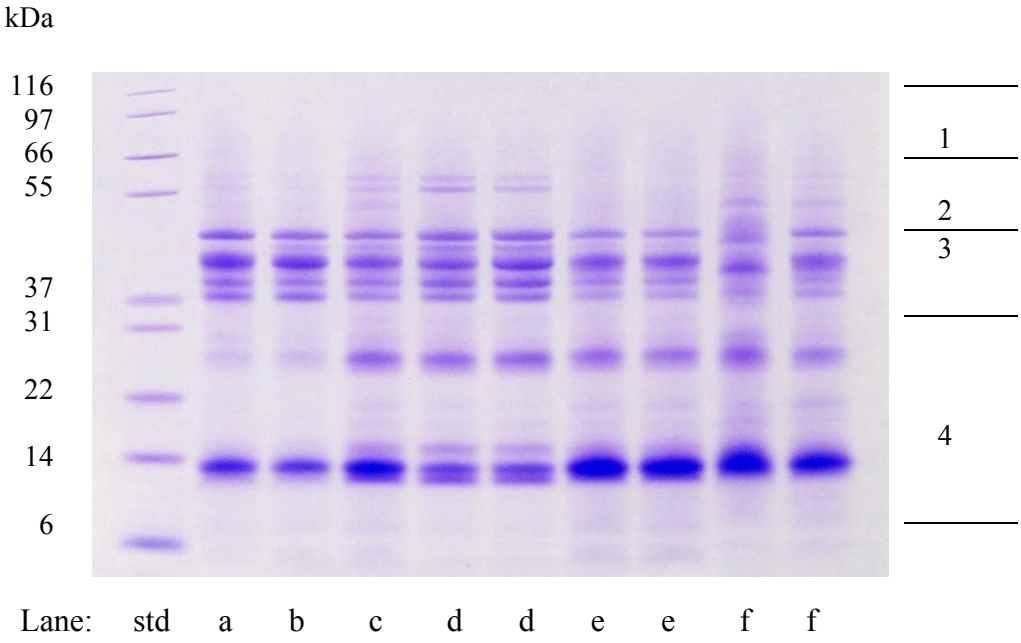


Figure 11.6 SDS-PAGE protein pattern of TX114 soluble protein from flour, gluten and the sequential acetic acid fractions

Notes		
	Lane a	Soft wheat flour (cv Rosella)
	Lane b	Hard wheat flour (cv Lang)
	Lane c	Gluten (from cv Lang flour)
	Lane d	0.01 M acetic acid soluble fraction
	Lane e	0.1 M acetic acid soluble fraction
	Lane f	Acetic acid insoluble fraction
	std	Protein standard
	1	Region of HMW-GS
	2	Region of ω-gliadin
	3 and 4	Region of α,β,γ-gliadins and LMW-GS

In a comparison of TX114 extractability between the sequential acetic acid fractions, the extraction yield is the highest for the 0.01 M acetic acid soluble fraction (2.72%, w/w of total protein), followed by 0.1 M acetic acid soluble fraction (1.74%) and the lowest at the acetic acid insoluble fraction (1.39%). This indicates that the TX114 extractability of protein decreases from the 0.01 M acetic acid soluble fraction to the 0.1 M acetic acid soluble fraction and the acetic acid insoluble fraction.

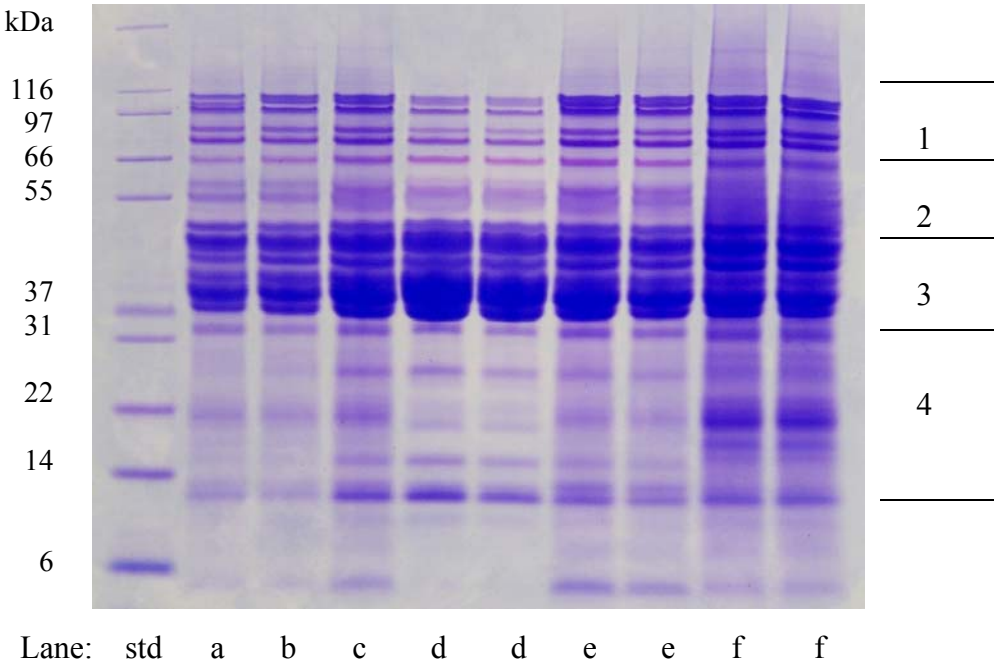


Figure 11.7 SDS-PAGE protein pattern of TX114 insoluble protein from flour, gluten and the sequential acetic acid fractions

Notes		
	Lane a	Soft wheat flour (cv Rosella)
	Lane b	Hard wheat flour (cv Lang)
	Lane c	Gluten (from cv Lang flour)
	Lane d	0.01 M acetic acid soluble fraction
	Lane e	0.1 M acetic acid soluble fraction
	Lane f	Acetic acid insoluble fraction
	std	Protein standard
	1	Region of HMW-GS
	2	Region of ω-gliadin
	3 and 4	Region of α,β,γ-gliadins and LMW-GS

The results of SDS-PAGE showed that the protein patterns of the TX114 protein extracts of the three fractions are similar (Figure 11.6, lanes d, e and f). In each case, the fractions consisted of several distinct protein bands including the 13 and 26 kDa proteins as well as gliadins in the region of MW of 37−55 kDa. However, the intensity of some protein bands varied. In the region of gliadins, a protein band at approximate MW of 44.7 kDa occurred in the extract of all three fractions. It was also found that protein bands at approximate MW values of 38.2, 40.2 and 48.6 kDa appeared at a high intensity in the TX114 extract of the 0.01 M acetic acid soluble fraction but were relatively faint in the TX114 protein extracts of the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction.

The amphiphilic proteins have been characterised on SDS-PAGE and it has been reported that the protein bands of PIN-a and PIN-b were not well separated (Morris et al., 1992). In the current study, in the region of amphiphilic proteins, two separate bands with approximate MW of 12 and 13 kDa were observed in the TX114 protein extract of the 0.01 M acetic acid soluble fraction but were not clearly seen in the TX114 protein extract of the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction (Figure 11.6, lanes d, e and f). In order to identify the presence of PIN-a and PIN-b in the TX114 protein extract of the three fractions, the CE method has been selected in this study as it has been used for most of studies of PIN in relation to the hardness of wheat grain (Day et al., 1999).

11.5.2 Identification of puroindolines in the Triton X114 protein extract

The protein profile of the TX114 protein extract was further analysed on CE to verify the presence of PIN-a and PIN-b in the sequential acetic acid fractions. As standards of these proteins are not available, the TX114 protein extract of soft wheat (Rosella cv) and hard wheat (Lang cv) were used to identify these peaks on the CE chromatogram in comparison with the literature (Day and Schofield, 2001). The CE profiles of TX114 protein extracts from soft and hard wheat flour are illustrated in Figure 11.8. Four main peaks on the CE profile of the TX114 protein extracts of wheat have been reported previously (Day and Schofield, 2001). These included the first two peaks with migration times of 5.7 and 5.9 min identified as purothionins and the third and fourth peaks with migration times of 6.6 and 7.5 min identified as PIN-b and PIN-a, respectively. It has been shown that wheat hardness is associated with mutations in PIN-b (Lillemo and Morris, 2000) and this is confirmed by the observations of the PIN-b peak height of hard wheat flour being relatively low compared to that for the soft wheat flour (Figure 11.8).

Based on the previous characterisation of PIN-a and PIN-b in the soft and hard wheat flour, PIN-a and PIN-b in the current study were identified as the peaks migrating at 8.46 and 7.35 min respectively; however, these peaks migrated more slowly compared to the migration times of 7.49 and 6.61 min, respectively, reported in the previous study (Day and Schofield, 2001). The difference in migration times could be due to the various sources of chemicals used in the CE analysis.

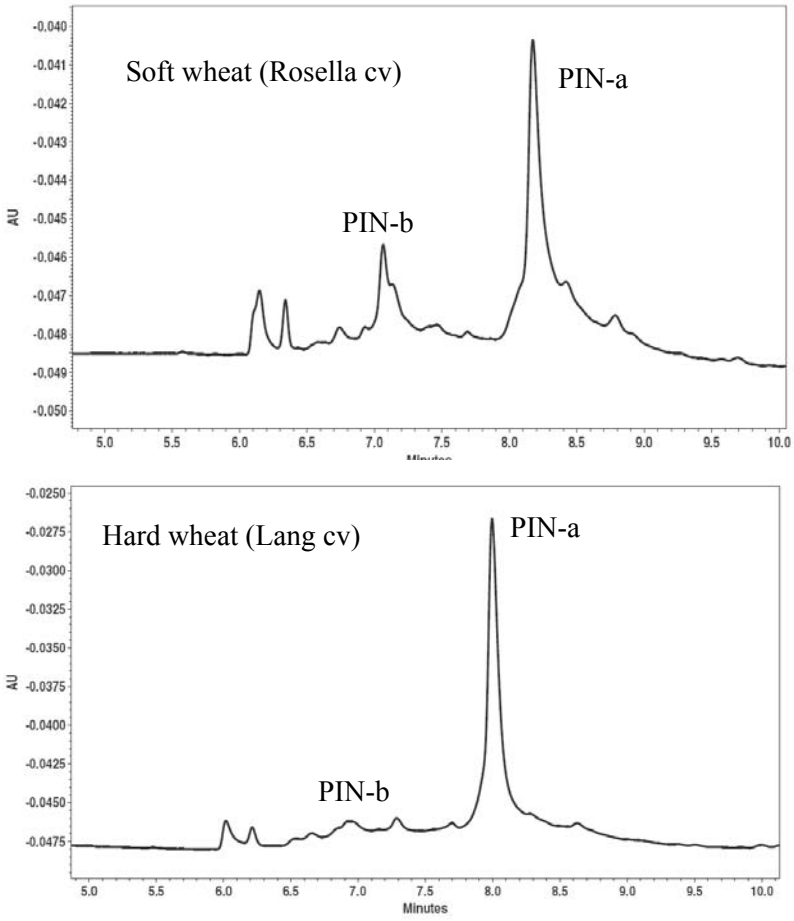


Figure 11.8 CE profiles of total Triton X114 protein extract from soft and hard wheat flour

The PIN-a/PIN-b CE migration time ratio was reported to be more reliable than the absolute migration time in the differentiation of endosperm texture (Day and Schofield, 2001). The results in the current study show that the PIN-a/PIN-b CE migration time ratios of the soft and hard wheat flours are close to the reference values (Table 11.4). It is also noted that the ratio of the peak areas for PIN-a and PIN-b in of the flour samples were in the range of the values reported for the TX114 protein extract of half-wheat-grains (Day et al., 2002). Based upon the observations (Table 11.4), the migration time of PIN-a and PIN-b and the ratio of the migration times can be used to identify the presence of the PIN components in the sequential acetic acid fractions.

Table 11.4 The ratios of migration times and peak areas for PIN-a and PIN-b from CE of flour and gluten

	Ratio of PIN-a/PIN-b			
	Peak area	Migration time	Peak area	Migration time
	Current study *[1]		Reference (Day et al., 2002)*[2]	
Soft wheat flour	2.4 ± 0.1	1.148	1.6–3.0	1.149
Hard wheat flour	5.7 ± 0.5	1.152	2.7–7.9	1.156

Notes *[1]: Soft wheat and hard wheat flours are from Rosella and Lang cultivars, respectively
*[2]: Soft wheat and hard wheat flours are from Riband and Hereward cultivars, respectively

The PIN-a and PIN-b ratios for the migration time and peak area were also calculated to evaluate the CE profile of the TX114 protein extracts from the three sequential acetic acid fractions (Table 11.5). When these ratios are compared to those of the gluten control, PIN-a and PIN-b appear to be present in all the fractions although the proportions vary. The ratios of PIN-a to PIN-b in the peak area reduced from the 0.01 M acetic acid soluble fraction to the 0.1 acetic acid soluble fraction and acetic acid insoluble fraction, demonstrating that the 0.01 M acetic acid soluble fraction contained relatively higher proportion of PIN-a than the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction. These were reflected in the CE chromatogram of the TX114 extracts, in which PIN-a was found in all the fractions whilst PIN-b occurred as a small peak in the 0.1 M acetic acid soluble and acetic acid insoluble fractions and was barely detectable in the 0.01 M acetic acid soluble fraction (Figure 11.9). This confirmed the results of SDS-PAGE of TX114 protein extracts in Figure 11.6, showing that protein bands at approximately 13 kDa in the TX114 extracts of both the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction were similar but different to that of the 0.01 M acetic acid soluble fraction.

In addition, two peaks following PIN-a and having migration times of 8.70 and 9.02 min occurred in substantial amounts in the TX114 extract of the 0.01 M acetic acid soluble fraction while they were not obvious in that of the other fractions. These peaks have not been reported in the literature. As these proteins exist in the TX114 protein

extract, they might have specific lipid-binding properties. Further purification and identification of these proteins, possibly by HPLC and mass spectroscopy, would be required to gain further understanding on their structures and functional roles.

Table 11.5 The ratios of PIN-a and PIN-b CE migration time and peak area of the sequential acetic acid fractions

	Ratios of PIN-a/PIN-b in	
	Peak area	**Migration time**
0.01 M acetic acid soluble fraction	15.0 ± 0.4	1.160
0.1 M acetic acid soluble fraction	4.4 ± 0.4	1.153
Acetic acid insoluble fraction	2.5 ± 0.3	1.156
Gluten (Lang)	4.9 ± 1.7	1.158

11.6 Effect of reducing agent on the free lipid distribution in acetic acid insoluble gluten fraction

In the previous sections, the protein composition of the sequential acetic acid fractions has been analysed and compared. This section is to focus on the effect of disulfide bonds on the interactions of protein and lipid in gluten. From the results of Chapter 10, glutenins were primarily found in the acetic acid insoluble fraction. The bound non-polar lipid found in this fraction could be entrapped within the polymeric structure of HMW-GS and LMW-GS that are stabilised by disulfide and hydrogen bonds (Shewry et al., 1992; Bushuk, 1998; Wrigley et al., 2006). Disulfide bonds would be disrupted by treatment of the acetic acid insoluble fraction with reducing agent (dithiothreitol). If there is any association of non-polar lipid and protein stabilised through disulfide-bond linkage, the use of reducing agent could convert the bound lipid to free lipid, hence resulted in an increase of total free lipid level.

The acetic acid insoluble fraction was treated with dithiothreitol at 50 mM and then dried under high vacuum conditions. The free lipid of treated and non-treated samples was extracted using petroleum ether and the lipid extracts were analysed using HPLC.

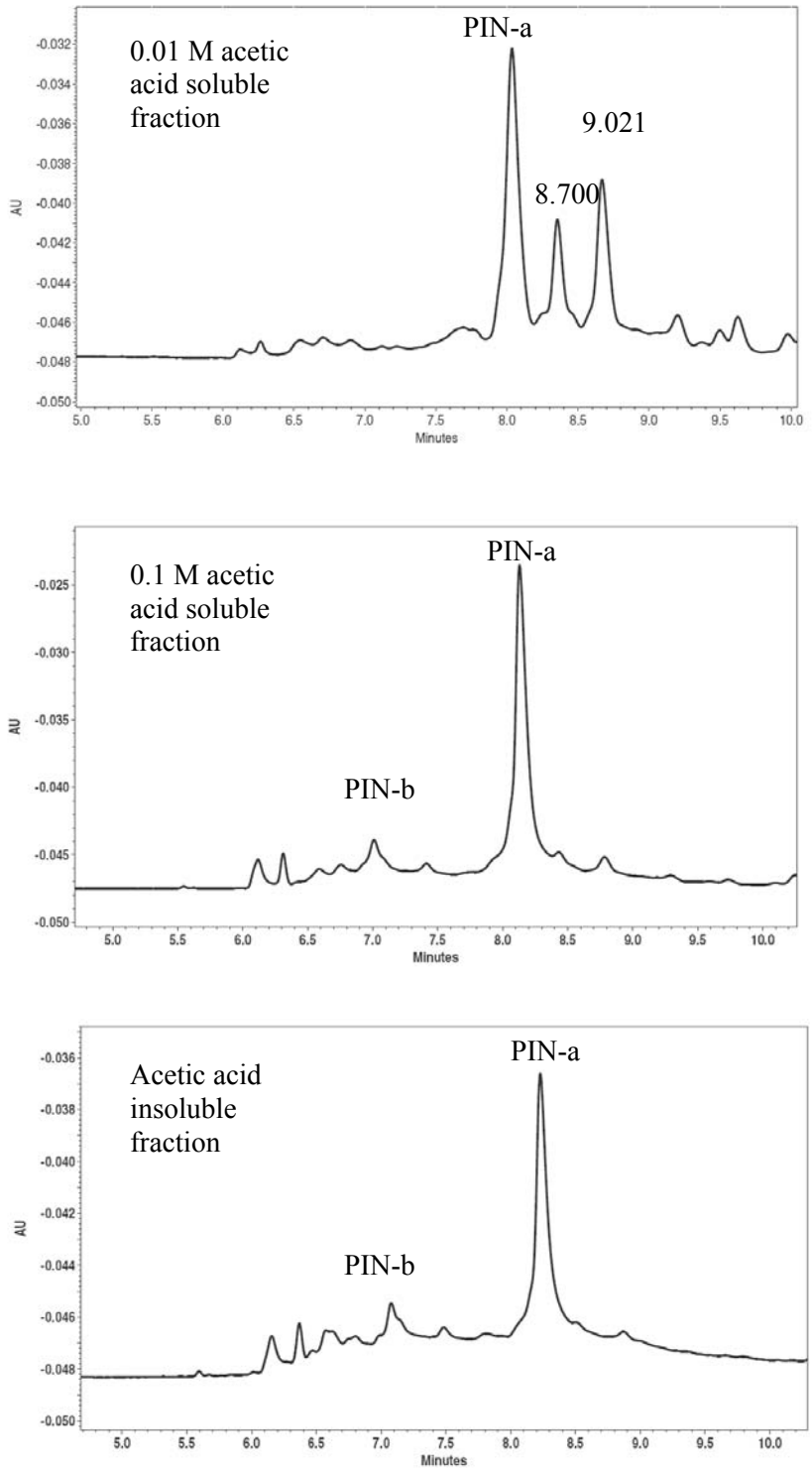


Figure 11.9 CE profile of Triton X114 protein extracts of the sequential acetic acid fractions

The results showed that the level of free lipid in the acetic acid insoluble fraction was not significantly changed after treatment with the reducing agent (Table 11.6). From the HPLC results, non-polar lipids are the only lipids presented in the free lipid extract (Figure 11.10). The results indicate that the disruption of disulfide bonds of protein in the insoluble fraction does not have a significant effect on the association of non-polar lipids and protein. The large number of hydrogen bonds occurring in gluten might have a strong influence on the interaction of proteins and lipids. This could involve either stimulation of hydrophobic interaction or entrapment of lipid within their structures. The current findings could be used to explain the results of the previous study on the occurrence of protein and lipid interactions in flour when adding water only (Davies et al., 1969). The mechanical mixing is to facilitate the formation of hydrogen bonds, thereby increasing the levels of lipid binding.

Table 11.6 Effect of dithiothreitol on level of free lipid in the acetic acid insoluble fraction

	Free lipid (%, w/w)	
	With dithiothreitol (50 mM)	**Without dithiothreitol**
Replicate 1	0.78	1.30
Replicate 2	0.55	0.62
Replicate 3	0.56	0.64
Average ± stdev	0.63 ± 0.13	0.85 ± 0.39

11.7 Preliminary study on identifying lipoproteins in acetic acid soluble gluten fractions using a native NuPAGE gel

Previous studies have indicated that the interaction between protein and lipid in gluten might involve lipoproteins in which glutenin is the protein component (Olcott and Mecham, 1947) or alternatively with lipid-binding proteins (Marion and Clark, 1995; Marion et al., 2003). These lipoproteins were recognised by the co-existence of lipid in protein extracts. Work in this area has been limited by the lack of a method to directly identify the presence of lipoproteins in gluten. However, there are methods reported for characterising lipoproteins in shrimp extract and in blood serum using electrophoresis

on native PAGE (Yepiz-Plascencia et al., 1995) and agarose gels (Greenspan et al., 1995). Based upon the underlying concept of these methods, preliminary studies have been carried out using a procedure for identifying lipoproteins and this has been applied to the acetic acid soluble gluten fractions.

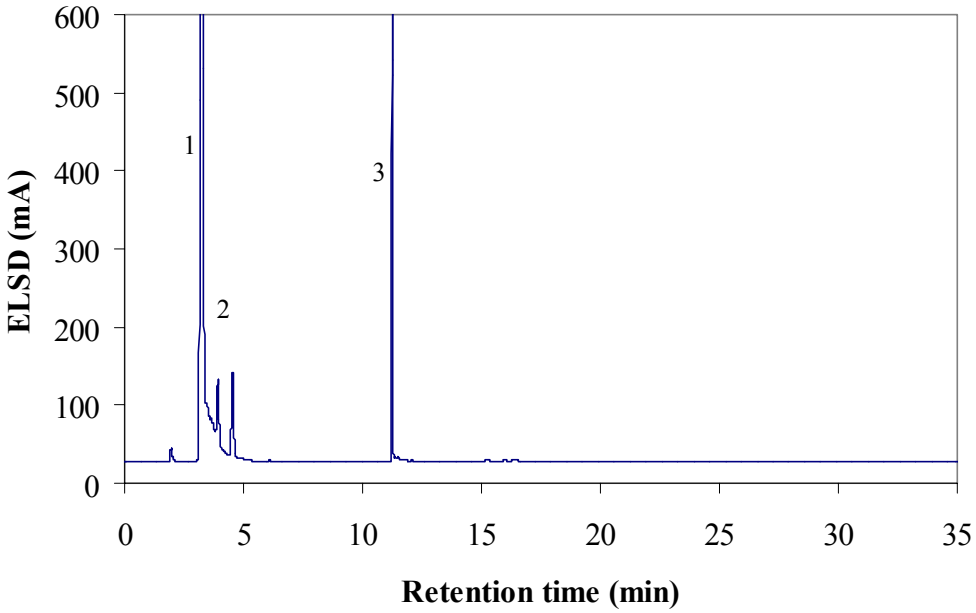


Figure 11.10 HPLC chromatogram of free lipid extract of dithiothreitol treated gluten

Notes 1 Triacylglycerol (TAG)
2 Diacylglycerol (DAG)
3 Monoacylglycerol (MAG) and solvent

In a series of trials, native NuPAGE gels have been used in conjunction with staining techniques for protein and lipid components in an attempt to identify lipoproteins in the 0.01 M and 0.1 M acetic acid soluble fractions from gluten. These acetic acid soluble fractions were solubilised using various combinations of sample buffer, reducing agent and salt. Following electrophoresis, the gel was firstly stained with Coomassie R-250 in order to visualise protein bands and finally with Sudan Black which is known to detect lipids and the results are shown in Figure 11.11.

The electrophoretic patterns show that a substantial amount of proteins in the 0.01 M acetic acid soluble fraction could enter the matrix of the native NuPAGE gel whilst a relatively small proportion of that in the 0.1 M acetic acid soluble fraction can (Figure 11.11). This indicates that some of proteins in the 0.01 M acetic acid soluble fraction

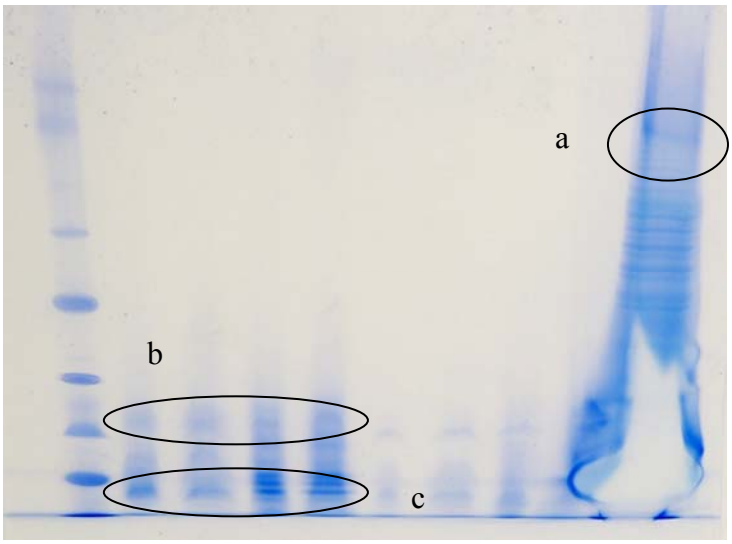
had relatively LMW while most of those in the 0.1 M acetic acid fraction were of larger size. The current experimental condition, therefore, might only be useful to detect lipoproteins in the 0.01 M acetic acid soluble fraction. The addition of salt into the sample buffer did not assist the solubilisation of protein, demonstrated by the unclear protein band occurring in the samples solubilised without and with salt (Figure 11.11, lanes 2 and 3 of gel I). The addition of reducing agent (dithiothreitol) to the sample buffer would be expected to break any disulfide bonds in the proteins, thereby reducing the size of the molecules and increasing their solubility. Consistent with this, higher amounts of protein were found to enter the native NuPAGE gel, demonstrated by more intensive protein bands appeared on the gel (Figure 11.11, lanes 4 and 5).

After lipid staining, the lipoproteins can be detected with a darker colour at the same position of protein band. The effectiveness of the staining procedure for lipoporoteins was validated through the use of lipoprotein standards. These can be seen on lane 9 of the gel, in which colour of a protein band was very slight on the protein stained gel (Figure 11.10, gel I, circle a) but very dark after staining with Sudan Black (Figure 11.10, gel II, circle a).

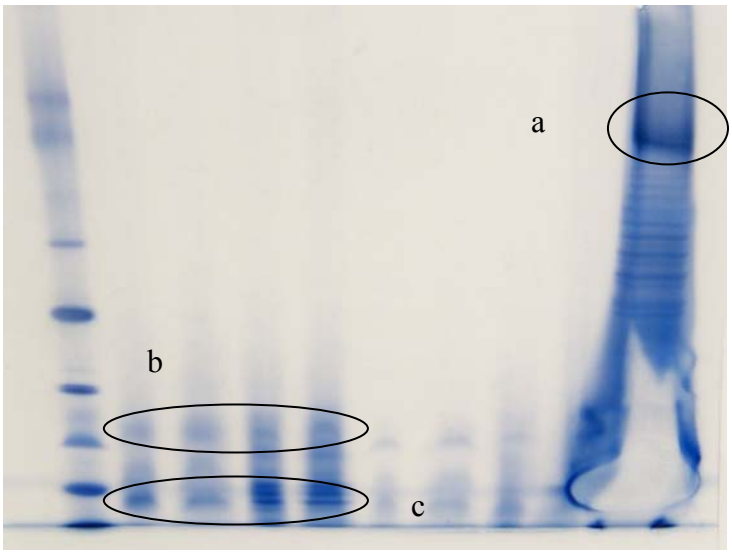
With all soluble fractions, protein bands were observed on the region designated as circle b and c on gel I. After staining with Sudan black, the colour of protein bands in the circle b did not change, however, protein bands in the circle c became more intense. This indicated the occurrence of lipoproteins in the 0.01 M acetic acid soluble fraction.

The apparent identification of lipoproteins under conditions where reducing agent was used in the buffer demonstrates that these lipoproteins probably associate with other proteins or themselves through disulfide bonds, as they required treatment with reducing agent in order to form clearly separate protein bands.

Gel I: Native Nu-PAGE gel with only protein stain (Coomassie)



Gel II: Native Nu-PAGE gel lipid stain (Sudan Black) after protein stain (Coomassie)



Lane 1 2 3 4 5 6 7 8 9

Figure 11.11 Native NuPAGE gel of the acetic acid soluble fraction with protein and lipid staining to detect lipoproteins

Notes The significance of regions designated as a, b and c is discussed in the text in Section 11.6

Lane 1	Protein standards at MW of 1236, 1048, 720, 480, 242, 146, 66 and 20 kDa from the top
Lane 2,3	0.01 M acetic acid soluble fraction without and with salt
Lane 4,5	0.01 M acetic acid soluble fraction treated with 50 mM dithiothreitol without and with salt
Lane 6,7	0.1 M acetic acid soluble fraction without and with salt
Lane 8	0.1 M acetic acid soluble fraction treated with 50 mM dithiothreitol without salt
Lane 9	Mixture of lipoprotein standards

11.8 Summary and general discussions

The amino acid composition and protein profile of RP-HPLC fractions of the three sequential acetic acid fractions indicates that the majority of proteins in the 0.01 M acetic acid soluble fraction are gliadin while glutenin occurred in both the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction. Of the LMW-GS and gliadins in the three fractions, it was observed that the concentrations of proteins at approximate MW of 38, 39, 40.8, 42.7, 44.7 and 49 kDa decreased and of 41.5, 45, 46.6, 52.4 and 53 kDa increased from 0.01 M acetic acid soluble fraction to the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction. The decrease of particular proteins might indicate the association of these with phospholipids, particularly LPE and LPC because the levels of these lipids also decreased (Section 10.5 and Figure 10.7). Likewise, the proteins at approximate MW of 41.5, 45, 46.6, 52.4 and 53 kDa might preferentially associate with glycolipids.

Puroindolines, lipid-binding proteins were found in higher amount in the 0.01 M acetic acid soluble fraction compared to the other fractions. The proportion of PIN-a/PIN-b was different between the three fractions. The 0.01 M acetic acid soluble fraction contained a relatively high proportion of PIN-a whilst the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction had relatively high proportion of PIN-b. On basis of the characterisation of PIN-a and PIN-b in interacting with polar lipids, PIN-a appears to be tightly associated with all wheat phospholipids including neutral charge lipid whilst PIN-b tends to interact with only negative charged phospholipids (Dubreil et al., 1997). The distribution of PIN-a and PIN-b in the three fractions could also explain the distribution of phospholipid components (Section 10.5.2). It was found that PS was present as a high proportion in the fraction containing a preponderance of PIN-b whilst a high level of LPC occurred in the 0.01 M acetic acid soluble fraction containing a high proportion of PIN-a. This could be attributed to PS being a negatively charged lipid while LPC has a net neutral charge (Harwood, 1994).

The reducing agent treatment used in conjunction with the detection of lipoproteins in the acetic acid soluble fractions and extracting free lipid in the acetic acid insoluble fraction has clearly demonstrated the effect of disulfide and hydrogen bonds on the

association of protein and lipid. The disruption of disulfide bonds in the 0.01 M acetic acid soluble fraction allowed some proteins to enter the native PAGE gel for lipoprotein detection. On the other hand, the free lipid level remain unchanged after treatment with dithiothreitol and this indicates that disulfide bonds did not have a strong effect on the protein-lipid interaction in the acetic acid insoluble fraction.

Chapter 12

General discussion and conclusions

12.1 Introduction

The literature review on protein-lipid interactions in dough and gluten indicated that the results from the previous studies have left a variety of questions unresolved. The discussion continues on the issue of whether the interactions between protein and lipid in gluten occur through physical entrapment or by the formation of specific bonds between particular components. In order to clarify these issues, acetic acid was selected and applied in this investigation for altering the gluten matrix and fractionating gluten proteins. Consequently, changes in the distribution of lipid and protein were observed and these are used as a basis for understanding and discussing the interactions of protein and lipid in gluten.

The discussions described in this thesis fall into four broad areas. These are:

1) The occurrence of lipid in flour and gluten;
2) Non-polar lipids in the gluten matrix;
3) Glycolipids in the gluten matrix; and
4) Phospholipids in the gluten matrix.

Following the discussion, a new model of protein-lipid interactions in gluten is proposed and overall conclusions are drawn.

12.2 The occurrence of lipid in flour and gluten

In the initial phase of this study, particular samples of flour and gluten were selected for investigation and the lipid content analysed, particularly in terms of the proportion of free and bound lipid present. The purposes of this series of experiments were firstly to verify the suitability of the lipid extraction method for the study and secondly to determine the lipid content of flour and gluten samples from hard and soft wheat samples for comparison with literature values. The total lipid contents of the flours and

the corresponding gluten samples prepared from these flours were 1.39–1.56% and 7.0–7.9%, respectively. These values were within the range of lipid contents reported previously for flour (1.4–2.0%) (Chung, 1986) and are similar to values for commercial gluten (4.2–7.6%) (Ponte et al., 2000) and lab-prepared gluten (5.0–10.0%) (Dill, 1925).

Although the lipid contents of flour samples in this study were similar to previous reports, substantial variations were found in the levels of free and bound lipids (Section 8.7). Flour samples in this study contained a higher proportion of free non-polar lipids (0.76–0.81%) but were lower in the amount of bound non-polar lipid (0.07%) compared to the values reported by Chung (1986) for free non-polar lipid (0.5–0.7%) and bound non-polar lipid (0.1–0.3%). These differences may reflect the use of different wheat varieties in the studies as well as the variations in wheat cultivars, growth locations and harvest time (Chung and Ohm, 2000). However, the ratio of free to bound lipid in gluten prepared from both soft and hard wheat flours in the current study was approximately 1:2, and this is consistent with findings from previous reports (Olcott and Mecham, 1947; Chung, 1986). In addition, it was found that approximately 65–75% of the lipid in the flours was recovered in the corresponding gluten samples, confirming the values reported by Olcott and Mecham (1947). The current study indicates that approximately 50–60% of the free lipid in flour became bound lipid in the gluten. These results are similar to the proportion of free lipid that became bound during the mixing of a dough (Olcott and Mecham, 1947).

Many previous studies have focused on analyses of total lipid, free and bound lipid contents of flour and gluten as well as the relative proportions of the various lipid classes. However, the results did not clearly show how the lipid composition changed when flour was processed to make gluten. In order to understand these changes, the current study included a comparison of lipid classes between flour and the corresponding gluten (Section 8.7) and it was found that almost all of the non-polar lipids originally in the flour were retained in the gluten representing a higher level of bound lipid. Approximately half of the glycolipids and only a small amount of phospholipids from flour were recovered in the gluten but all occurred in the bound form. The differing levels of retention of non-polar lipid, glycolipid and phospholipid in gluten could relate to the specific properties of these lipid classes as well as the formation of the gluten matrix.

During gluten preparation, the hydration of flour could facilitate the formation of a number of hydrogen bonds within the gluten matrix (Bushuk, 1998; Wrigley et al., 2006). In addition, non-polar lipids have low polarity; therefore they tend to associate with other materials also having low polarity in the aqueous conditions. In this case, non-polar lipids may preferentially associate with gluten proteins, which are known to have a low net charge (Hoseney, 1998a), and this association might occur through hydrophobic interactions. The combination of hydrogen bond formation and hydrophobic interactions between non-polar lipids and gluten proteins could lead to the entrapment of these lipids within the gluten matrix when they associate with some of the gluten proteins during gluten preparation. This is consistent with the observation that most non-polar lipids originally in flour remained in the gluten.

The polarities of glycolipids and phospholipids are higher than that of non-polar lipids. Furthermore, phospholipids, are known to be starch lipids, having a strong association with starch granules in flour (Morrison, 1988). They can be removed during the starch washing process, which is an explanation for the relatively small amounts of phospholipids remaining in gluten. The amount of bound glycolipids in gluten was similar to that in flour, which might suggest that glycolipids did not further interact with gluten components during gluten preparation. However, a previous study indicated that all polar lipids in flour became bound at the first stage of dough mixing (Chung and Tsen, 1975a). The free glycolipids which became bound during dough mixing could be either removed with starch and soluble materials during the gluten recovery or partially retained in gluten structure. Further study is required to provide evidence on the source of those glycolipids which remain associated with the gluten.

In summary, the relatively large amount of non-polar lipid and the small proportion of glycolipids and phospholipids remaining in gluten can be attributed to the entrapment of these lipids during gluten matrix formation as well as the association of these lipids with specific protein. The remaining lipids in gluten could relate to the association of these lipids with gluten components. In order to further investigate these observations, the next phase of this study involved the treatment and fractionation of gluten with acetic acid solutions, followed by analysis of the lipid components. This provides a basis for the discussion of the interactions of individual lipid class in the gluten matrix.

12.3 Non-polar lipids in the gluten matrix

In the experimental data reported in this study, the acetic acid treatment and fractionation of gluten have shown an effect of increasing the solubility of gluten protein and an alteration of the free and bound lipid distribution. The changes primarily occurred for the non-polar lipid, demonstrating that the level of free non-polar lipid was increased and consequently the amount of bound lipid is reduced after acetic acid treatments (Table 9.2). For the fractionation studies, with both the single level and also the sequential approach using acetic acid at two concentrations, it was also found that the level of free non-polar lipid increased compared to the gluten control (Tables 9.6, 10.2 and 10.4). In the single acetic acid fractionation, the majority of free non-polar lipid was retained in the pellet at low acetic acid concentration (0.01 M) but at higher acetic acid concentrations (0.05 or 0.1 M), it was found in the soluble fractions (Table 9.5). In the sequential acetic acid fractionation, a high proportion of this lipid was also found in the 0.1 M acetic acid soluble fraction (Table 10.5). The increasing amounts of free non-polar lipids in gluten under acetic acid treatments and fractionation have not previously been reported although many groups have indicated that lipids preferentially associate with protein solubilised in an acetic acid solution (Chung and Tsen, 1975a; Frazier et al., 1981).

In considering the increased amount of free lipid following acetic acid treatment and fractionation found in the current investigation, this was initially thought to be due to partial solubility of lipid in aqueous solution although lipid is known to be sparingly soluble in water. The solubility of non-polar lipids, glycolipids and phospholipids in an aqueous condition has not been reported (Larsson and Quinn, 1994). In an aqueous environment, non-polar lipids lack the ability to transfer to an aqueous phase while polar lipids appear to interact with water to give a lamellar liquid-crystalline phase at a concentration greater than 50% (Larsson, 1985). Hence, if polar and non-polar lipids are present in the same proportion, the polar lipids tend to disperse in the aqueous solution at higher concentrations than for non-polar lipids. This effect was not observed in the distribution of free lipid after sequential acetic acid fractionation. Of the total lipid in the soluble fractions, polar lipids exist at a higher proportion than for non-polar lipids (Table 10.6). On the other hand, non-polar lipids appear as the predominant lipid class

in the free lipid and polar lipids occur as the major component of the bound lipid (Figure 10.4). Although the free lipid of the 0.1 M acetic acid soluble fraction (from the sequential acetic acid fractionation) and the 0.1 M acetic acid supernatant (from a single acetic acid fractionation) contain non-polar lipids, glycolipids and phospholipids, polar lipids (glycolipids and phospholipids) occur as a lower proportion than the non-polar lipid. This is evidence that the amount of free lipid occurring in the soluble fractions cannot be attributed to either the solubility or the dispersion of lipid in the aqueous solution.

During gluten preparation, non-polar lipids became associated with other gluten components, particularly gluten protein and it is likely that a number of hydrogen bonds form in the gluten matrix (Section 12.2). Under treatment or fractionation with acetic acid, some of these hydrogen bonds can be disrupted, facilitating the re-orientation of the gluten structure (Wrigley et al., 2006). The results of acetic acid treatment (Section 9.2) indicate that the change of gluten structure allows some of the bound non-polar lipids to become accessible to the process of solvent extraction.

The application of a single acetic acid fractionation at a low concentration (0.01 M) resulted in the removal and solubilisation of some of the gliadin components from the gluten network. Consequently, the non-polar lipids, which would normally be held within the gluten matrix, remain associated with the glutenin molecules in the pellets and as a result they could be extracted as free lipid (Section 9.3).

At higher acetic acid concentrations (0.05 and 0.1 M), glutenins became more soluble and this could be attributed to the change in their net-charge. Non-polar lipids remaining on the surface of protein molecules therefore were co-extracted with glutenin in the supernatant. This suggests that these lipids, usually held within gliadin and glutenin in the gluten matrix, preferentially associate with glutenin in the aqueous conditions.

In addition, the results of the sequential acetic acid fractionation indicate that non-polar lipid and glycolipids were the main components of the bound lipids in the 0.1 M acetic acid soluble and acetic acid insoluble fractions (Section 10.4), indicating the association of these lipids with glutenin, and this is consistent with previous reports (Chung and

Tsen, 1975a, 1975c; Frazier et al., 1981; Bushuk, 1985; Chung, 1986). However, in the current study there was evidence indicating that non-polar lipids and glycolipids interact with glutenin in a different way. Non-polar lipids were found to distribute evenly between free and bound lipid extracts of gluten fractions and therefore could be associated with glutenin through hydrophobic interactions in a non-specific way. On the other hand, glycolipids were found at a high proportion in bound form whilst only a very small amount occurred in the free form for the 0.1 M acetic acid soluble fraction. This indicates that glycolipids associate with gluten protein in a specific way, which is discussed in the following section.

Previously it has been found that glutenin present in the gluten matrix as polymeric proteins formed a network between HMW-GS and LMW-GS through inter and intra-molecular disulfide covalent bonds (Shewry et al., 1992). The current study found that non-polar lipid associates with glutenin in a non-specific way and the 0.1 M acetic acid insoluble fraction contained primarily glutenin. This fraction was treated with reducing agent in order to clarify whether these disulfide bonds would have any affect on the interaction of non-polar lipid with glutenin. The results presented in Section 11.6 indicate that the disruption of disulfide bonds does not have a significant effect on the association of non-polar lipids and glutenin.

In summary, in the current study it has been found that non-polar lipids associate with glutenin through hydrophobic interactions in a non-specific way. The increased amount of bound non-polar lipids during gluten preparation could be attributed to the formation of hydrogen bonds between gliadin and glutenin. This structure partially entrapped non-polar lipid within the matrix formed by these two proteins. This finding is consistent with the conclusions from physical testing using phosphorus magnetic resonance spectroscopy and freeze-fracture electron microscopy applied to wheat gluten (Marion et al., 1987). In the current study there was also evidence that specific lipids were held within the gluten structure. This confirms the results of a previous study on the occurrence of protein and lipid interactions in flour (Davies et al., 1969). As the formation of hydrogen bonds plays an important role in the interactions of non-polar lipids with gluten, the conditions, particularly water addition and mechanical mixing, are able to facilitate the formation of these bonds, thereby increasing the extent of lipid binding.

12.4 Glycolipids in the gluten matrix

Glycolipids were found to occur predominantly as bound lipid in gluten treated and fractionated with acetic acid (Tables 9.2, 9.5 and 10.5). It is noted that very small amounts of free glycolipids were found in the treated gluten and soluble fractions at 0.1 M acetic acid. These results indicate that acetic acid had a minimal effect on the interaction of glycolipids with gluten proteins. In the single acetic acid fractionation, the amounts of glycolipids increased corresponding with the higher protein content, primarily glutenin in the supernatant (Figure 9.9, B). This indicates that glycolipids preferred to associate with glutenin and this confirms the earlier reports of Chung and Tsen (1975a, 1975c). However, the current findings are at variance with the data presented by Bushuk (1985), who suggested that glutenin associated with polar lipid, particularly phospholipids. This could be due to the use of alcohol in the previous report (Bushuk, 1985) whilst acetic acid was applied in the current study for preparing glutenin and gliadin fractions. As a solvent, the polarity of alcohol is relatively high (Reichardt, 1988), hence it could have a strong effect on the solubilisation of polar lipids, thereby influencing the lipid extractability.

Furthermore, the sequential acetic acid fractionation showed that a large amount of glycolipids occurs as bound lipid in the 0.1 M acetic acid soluble and acetic acid insoluble fractions whilst only a small amount is present in the free lipid extract of the 0.1 M acetic acid soluble fraction (Figure 10.4). The increasing solubility of glutenin was observed at high acetic acid concentration corresponding with the appearance of free glycolipids. The results indicate the entrapment of glycolipids within polymeric glutenin molecules. If this actually occurred in the gluten matrix, the effect of acetic acid on producing free glycolipids would be expected in both soluble and insoluble fractions. However, free glycolipids were only found in the 0.1 M acetic acid soluble fraction. It is therefore suggested that rather than entrapment, the observations on extractability of the glycolipids indicates a specific association with glutenin.

Glycolipids can interact with protein through hydrophobic interactions as well as hydrogen bonds (Hoseney et al., 1970a). As acetic acid is able to disrupt hydrogen bonds or change the surface charge of proteins, the hydrogen bond between glycolipids

and glutenin might be weakened, and so the glycolipids can then be extracted with petroleum ether, a solvent of low polarity. This hypothesis was further strengthened by the absence of glycolipids in the free lipid extract of the acetic acid insoluble fraction and this fraction contains the protein which shows lower impact of the acetic acid on the surface charge and hydrogen bonds. The evidence supports the occurrence of interactions between glycolipids and glutenins within the structure of the gluten matrix.

In addition, glycolipids interact with glutenins occurring in either soluble or insoluble form, as demonstrated by the presence of similar amounts of bound glycolipid components per gram of protein (Figure 10.7). This is further confirmed by the similar ratio between MGDG and DGDG in both 0.1 M acetic acid soluble and acetic acid insoluble fractions (Figure 10.7 and Table 10.6). Interestingly, the ratio of MGDG and DGDG in the free lipid extract of the 0.1 M acetic acid soluble fraction is the same as in the bound lipid extract. This evidence also supports the possibility that glutenins and glycolipids associate through hydrophobic interactions and hydrogen bonds.

Further characterisations of protein in the three sequential acetic acid fractions (Chapter 11) found that glutenin was a predominant protein in the fraction containing a high proportion of glycolipid. This supports the conclusion on the interactions of glycolipids and glutenin within the gluten matrix. Moreover, the RP-HPLC results have indicated that the LMW-GS and gliadins at approximate MW of 41.5, 45, 46.6, 52.4 and 53 kDa might be directly involved in these interactions because their concentrations in the three acetic acid fractions were distributed in a pattern similar to that of the glycolipid contents. However, further studies on the interactions of these proteins with glycolipids are required to confirm these observations.

12.5 Phospholipids in the gluten matrix

Similar to the pattern found for glycolipids, phospholipids were also present as bound lipid in gluten treated and fractionated with acetic acid (Tables 9.2, 9.5 and 10.5). However, unlike the glycolipids, phospholipids occurred at a relatively high level in the fractions having a high proportion of monomeric proteins. This was observed in the 0.01 M acetic acid supernatant from the single acetic acid fractionation as well as in the

0.01 M acetic acid soluble fraction from the sequential acetic acid fractionation. The monomeric proteins were identified as gliadins through SE-HPLC (Figures 9.5 and 10.1), SDS-PAGE (Figures 9.4 and 10.2) and other approaches to characterisation described in Chapter 11. These results suggest that phospholipid preferentially associates with gliadins in the gluten matrix. It is noted that this observation is inconsistent with the previous report (Bushuk, 1985) that indicated the interaction of phospholipids and glutenin. As discussed in Section 12.4, the different findings is probably due to the variation in the method used for fractionation of glutenins and gliadins.

Furthermore, from the sequential acetic acid fractionation, it was observed that the levels of phospholipid decreased from the 0.01 M acetic acid soluble fraction to the 0.1 M acetic acid soluble and insoluble fractions while the densities of protein bands at MW of approximately 12, 16, 26.8 and 42 kDa also decreased (Figure 10.3 and 10.4). As discussed in Chapter 10, these proteins had a similar MW with some proteins found in the S protein fraction (Zawistowska et al., 1986), PIN (Blochet et al., 1991) and CM protein (Meredith et al., 1960). These particular proteins are known to associate with polar lipid and to have a high affinity for phospholipids and glycolipids (Zawistowska et al., 1986; Dubreil et al., 1997; Kooijman et al., 1997; Carr et al., 1992). This provides evidence that the 0.01 M acetic acid soluble fraction contains a number of proteins which are able to associate with phospholipids. Hence, the high level of phospholipids in the fractions containing a high proportion of gliadins could be attributed to the interactions of these lipids either with gliadins or lipid binding proteins.

When the lipid binding proteins in the sequential acetic acid fractions were characterised, a high level of PIN was found in the 0.01 M acetic acid soluble fraction compared to the other fractions. A relatively high proportion of PIN-a was found in the 0.01 M acetic acid soluble fraction whilst the 0.1 M acetic acid soluble fraction and the acetic acid insoluble fraction contained high levels of PIN-b. It was also found that PS, a negatively charged lipid (Harwood, 1994) was present as a high proportion in the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction containing a high proportion of PIN-b. On the other hand, a high level of LPC, a lipid having a net neutral charge (Harwood, 1994) occurred in the 0.01 M acetic acid soluble fraction which also contained a high proportion of PIN-a (Figure 10.5). These findings were consistent with

the ability of PIN-a to associate with all wheat phospholipids including those with neutral charge whilst PIN-b is able to interact with only negative charged phospholipids (Dubreil et al., 1997).

In addition, the characterisation of the sequential acetic acid fractions in Chapter 11 using the combination of RP-HPLC and SDS-PAGE showed that the LMW-GS and gliadins at approximate MW values of 38, 39, 40.8, 42.7, 44.7 and 49 kDa, decreased from 0.01 M acetic acid soluble fraction to the 0.1 M acetic acid soluble fraction and acetic acid insoluble fraction. The levels of phospholipids also decreased following a similar trend in those acetic acid fractions. These results indicate that phospholipids might also have specific interactions with these LMW-GS and gliadins. Further investigations are required to confirm these observations.

12.6 A newly proposed model of protein-lipid interactions in gluten

Research over a number of decades has shown that during dough mixing and gluten preparation, there are interactions between the glutenin and gliadin proteins which contribute to the formation of the gluten matrix. Based upon the previous work, a variety of models have been proposed to describe the protein interactions within the gluten structure. The most recent of these which reflects existing knowledge has been that proposed by Lasztity (1996), and in this polymeric glutenins form large molecules through the formation of disulfide bonds whilst gliadins interact with polymeric glutenins and other gliadins through hydrophobic interactions and hydrogen bonds. The original model of protein interactions is presented in Figure 12.1 as this has been used as the basis for a newly proposed model of protein-lipid interactions.

In relation to the Lasztity model of gluten structure it is noted that subsequent work has provided further confirmation of its validity. This includes the reports from Shewry and Tatham (1997) on the formation of disulfide bonds between LMW-GS and HMW-GS in polymeric glutenins as well as the hydrogen bonding and hydrophobic interactions between glutenins and gliadins (Wrigley et al., 2006). In addition, similar structural models of wheat gluten have been proposed by Belton (1999) and Shewry et al. (2001). In the Belton model, HMW-GS appear as linear polymers and play a role in the

viscoelastic properties whilst gliadins are presented as globular polymers and contribute to the formation of a viscous environment (Belton, 1999). The Lasztity model is closer to the Shewry model, in which HMW-GS provide a disulfide-bonded backbone, interacting with LMW-GS by disulfide bonds and with gliadins through non-covalent interactions (Shewry et al., 2001).

The Lasztity model of gluten structure has been used as a convenient starting point in the development a new model of the interactions between proteins and lipids within the gluten matrix. The findings in this study on the various interactions of lipid and gluten protein components have been used as the basis for proposing the model presented in Figure 12.2. The features shown in the model are that non-polar lipids preferentially associate with glutenin through hydrophobic interactions and some of them are entrapped in the matrix formed between glutenin and gliadin (Section 12.3). In addition, glycolipids and phospholipids associate with gluten proteins in a specific way. Glycolipids tend to associate with glutenin through hydrophobic interactions and hydrogen bonds (Section 12.4) whilst phospholipids preferentially interact with gliadins through hydrogen bonds (Section 12.5).

In previous decades, some models of protein and lipid interactions in dough systems have been proposed (Hoseney et al, 1970a; Wehrli and Pomeranz, 1970) and these were presented in Figure 5.1. The Hoseney model indicated the interactions of glycolipids with gliadin through hydrophilic links and with glutenins through hydrophobic bonds (Hoseney et al, 1970a). The alternative model revealed the presence of starch-glycolipid-gluten complex in dough (Wehrli and Pomeranz, 1970). The new model proposed in this study was specifically established from studies of the gluten matrix, primarily containing glutenins and gliadins as protein components and non-polar lipids, glycolipids and phospholipids as lipid components. The previous models indicated the interactions of only glycolipids with other components whilst the model proposed in this study describes interactions between various lipid classes and proteins within gluten matrix. It also indicates that different types of lipid classes tend to form specific interactions with particular gluten proteins.

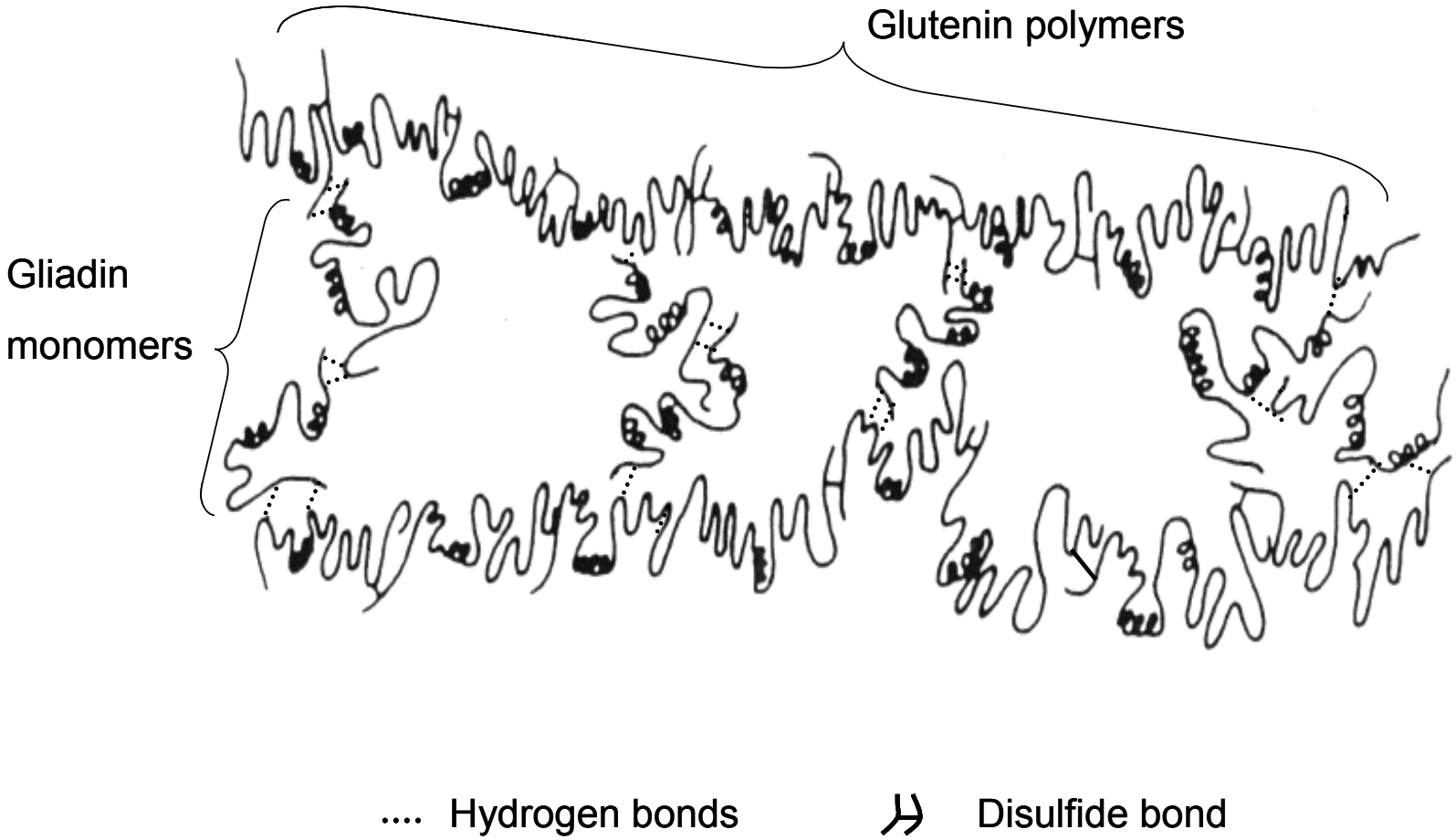


Figure 12.1 The model of gluten structure proposed by Lasztity and describing the arrangement of gliadin and glutenin components in the gluten matrix

Note The source of this model was Lasztity (1996)

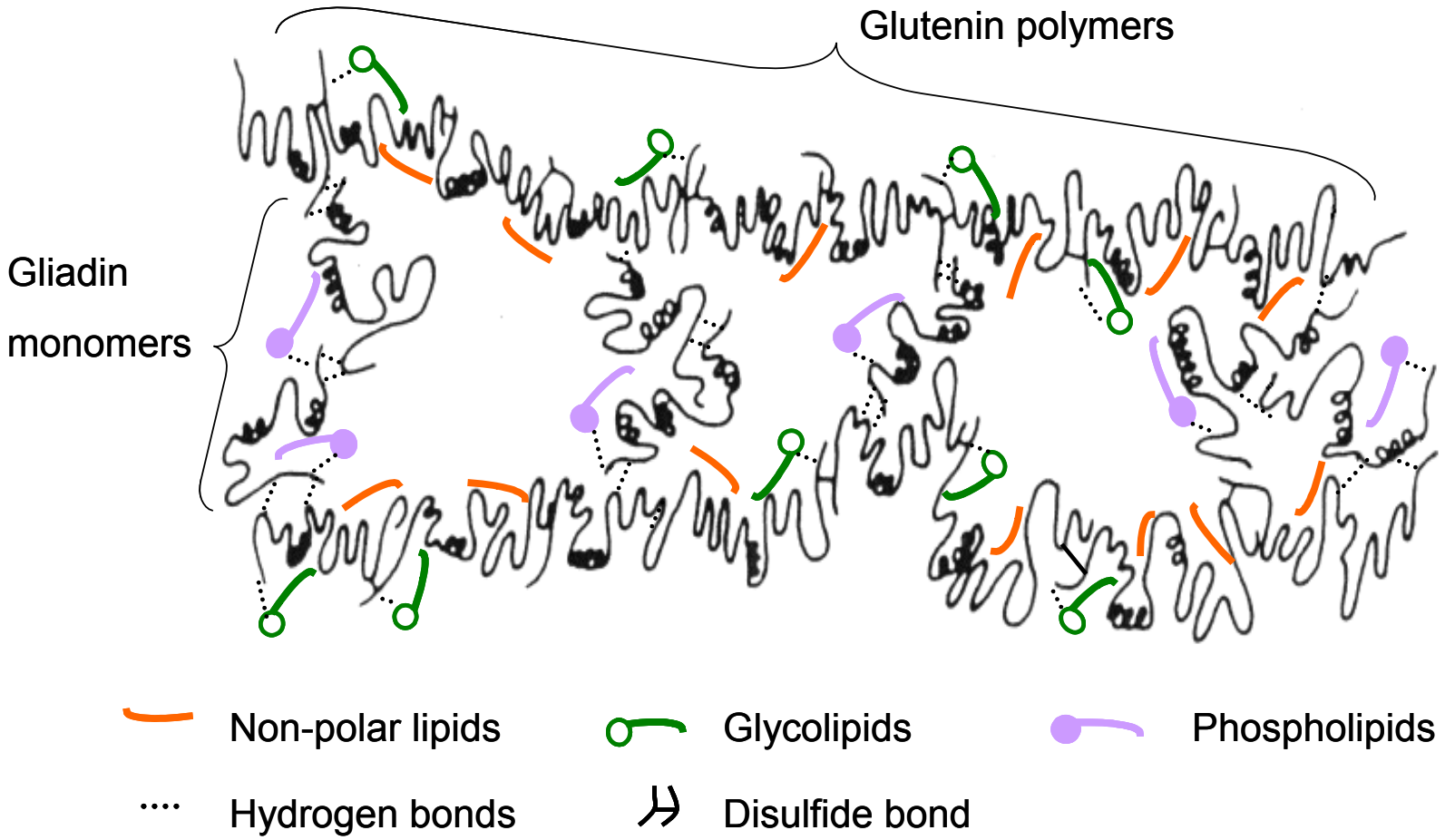


Figure 12.2 Proposed model of protein-lipid interactions in gluten based on the gluten interactions described by Lasztity (1996)

12.7 Summary of final conclusions

The investigations of the lipid extractability in flour and gluten and the distributions of lipid and protein in gluten treated and fractionated with various acetic acid concentrations have led to the following conclusions:

1) During gluten preparation, most of the non-polar lipids originally present in the flour were retained in the gluten with a corresponding increase in the level of the bound form. Half of the amount of glycolipids and a small proportion of phospholipids remained in gluten and all of these existed as the bound form. The retention of non-polar lipids in the gluten structure could relate to the association of non-polar lipids with gluten components and the formation of hydrogen bonds within the gluten matrix.

2) Upon treatment of gluten with acetic acid solutions, there was an effect on the hydrogen bonds formed between gliadin and glutenin components in the matrix. This resulted in separation of these proteins at a relatively low concentration of the acid (0.01 M). These effects on gluten proteins corresponded with increases in the level of free non-polar lipids in the gluten.

3) It was found that non-polar lipids associated with glutenin through hydrophobic interactions in a non-specific way. When associating with glutenin, these lipid components were entrapped or locked within the gluten matrix which forms as a result of hydrogen bonds between glutenin and gliadin contributing to the structure and integrity of the matrix.

4) The evidence from this study indicates that glycolipids associate with glutenin through hydrophobic interactions and hydrogen bonds. On the other hand, the results do not provide any indication that glycolipids are associating with gliadins.

5) Phospholipids were found to occur in higher proportions in those protein fractions containing high levels of gliadins. This demonstrates that phospholipids probably interact with either gliadins or lipid binding proteins within the gluten matrix.

6) On the basis of the comprehensive studies reported here, a new model of the interactions between lipid and protein components within the gluten matrix has been developed and proposed.

12.8 Possible areas for future research

From the current study it was found that lipids associated with gluten protein in both non-specific and specific ways. In addition, as the gluten matrix is stabilised with many non-covalent bonds, particularly hydrogen bonds, some lipids are also locked within the three dimensional structure formed by the matrix. Accordingly, it would be impossible to remove all lipids from gluten without a resultant loss in functionality. However, one of the fractionation approaches used in this study, the sequential acetic acid fractionation, was able to produce protein fractions having a low level of lipid (specifically the 0.01 M acetic acid soluble fraction). This could be an alterative approach allowing the production of a gluten product containing a low level of lipid. Additional studies would be required to establish the feasibility and potential for scaling up tis approach. However, the possibility that this might minimise some of the problems of deterioration in flavour and functionality encountered during storage of commercial gluten products warrants further investigation.

The amount of bound glycolipids in gluten was found here to be similar to that in the flour. One previous study indicated that all polar lipids became associated with gluten proteins at the first stage of dough mixing. Further investigation on the source of glycolipids remaining in the gluten could add further understanding on the interaction of glycolipids with protein and starch as well as explain the fact that acetic acid had a minimal effect on the level of bound glycolipids found in gluten.

Glycolipids and phospholipids have been found to associate with gluten proteins through hydrophobic interactions and hydrogen bonds. Further studies using synchrotron equipment, particularly X-ray light scattering could be applied to establish the structural components specifically involved in protein-lipid interactions as well as identifying the binding sites of lipid and protein. In addition, glycolipids and

phospholipids preferentially associate with specific proteins; therefore, the study of the effect of these lipids on modifying the functional properties of gluten could be of benefit for increasing the range of practical gluten applications.

The sequential acetic acid fractionation produces gluten fractions having a range of lipid and protein contents. The functional properties of these fractions could be of particular interest and assist in the interpretation of the various associations of lipid and protein. In addition, the results of rheological studies on these fractions could be useful for extending the applications of gluten in the food industry.

Phospholipids were found in the fraction containing high level of gliadins as well as lipid-binding proteins. The study of the interactions of lipid-binding proteins and gliadins in the gluten matrix could be useful in explaining the co-existence of phospholipids with gliadins and lipid-binding proteins. This could provide more knowledge on the role of lipid-binding proteins in gas cell formation and their interaction with other components thereby contributing to the texture of bread.

In the current study, it was also found that some of the LMW-GS and gliadins associate with glycolipids and others interact with phospholipids. However, the comprehensive characterisation of these proteins was beyond the scope of this project. Further studies on LMW-GS and gliadins in relation to the association of polar lipids could provide more specific information on the interactions of these protein components with lipids.

Finally, whilst much remains for further investigation, it is hoped that the studies reported here provide a basis both for enhanced utilisation of gluten as well as for future studies of this fascinating and complex food ingredient.

References

Note In this thesis referencing has followed the recommendations found in the Journal of Cereal Science (see JCS 2006 below).

AACC, 1995a. Method 30-10: Crude fat in flour, bread, and baked cereal products not containing fruit. Approval Methods of the AACC, 9th ed. The Association, St. Paul, Minnesota.

AACC, 1995b. Method 44-15: Moisture: Air-Oven Methods. Approval Methods of the AACC, 9th ed. The Association, St. Paul, Minnesota.

AACC, 1995c. Method 46-30: Crude Protein-Combustion Method. Approval Methods of the AACC, 9th ed. The Association, St. Paul, Minnesota.

Amiour, N., Merlino, M., Leroy, P., Branlard, G., 2002. Proteomic analysis of amphiphilic proteins of hexaploid wheat kernels. Proteomics 2, 632-641.

Anderson, O.D., Hsia, C.D., Torres, V., 2001. The wheat gamma-gliadin genes: characterization on ten new sequences and further understanding of gamma-gliadin gene family structure. Theoretical and Applied Genetics 103, 323-330.

Australian Bureau of Statistics, 2006. 1301.0 - Year Book Australia. [On-line Internet]. Available: http://www.abs.gov.au/ausstats/abs@.nsf/Latestproducts, ABS. Accessed 24 August 2006.

Bailey, C.H., 1941. A translation of Beccari's lecture 'concerning grain' (1728). Cereal Chemistry 18, 555-561.

Batey, I.L., 2004. Gluten and modified gluten. In: Wrigley, C.W., Corke, H., Walker, C.E. (Eds.), Encyclopedia of Grain Science, Volume 2. Elsevier Academic Press, Oxford, pp. 25-30.

Batey, I.L., Gupta, R.B., MacRitchie, F., 1991. Use of size-exclusion high-performance liquid chromatography in the study of wheat flour proteins: An improved chromatographic procedure. Cereal Chemistry 68, 207-209.

Bean, S.R., Bietz, J.A., Lookhart, G.L., 1998. High-performance capillary electrophoresis of cereal proteins . Journal of Chromatography A 814, 25-41.

Bean, S.R., Lookhart, G.L., 2000. Electrophoresis of cereal storage proteins. Journal of Chromatography A 881, 23-36.

Bean, S.R., Lookhart, G.L., 2003. Separation of gluten proteins by high performance capillary electrophoresis. In: Shewry, P.R., Lookhart, G.L. (Eds.) Wheat Gluten Protein Analysis. American Association of Cereal Chemists, St. Paul, Minnesota, pp 91-113.

Bekes, F., Zawistowska, U., Zillman, R.R., Bushuk, W., 1986. Relationship between lipid content and composition and loaf volume of twenty-six common wheats. Cereal Chemistry 63, 327-331.

Bell, B.M., Daniels, D.G.H., Fearn, T., Stewart, B.A., 1987. Lipid composition, baking qualities and other characteristics of wheat varieties grown in the UK. Journal of Cereal Science 5, 277-286.

Bell, B.M., Daniels, D.G.H., Fisher, N., 1979. The effects of pure saturated and unsaturated fatty acids on breadmaking and on lipid binding, using Chorleywood Bread Process doughs containing a model fat. Journal of the Science of Food and Agriculture 30, 1123-1130.

Belton, P.S. 1999. Mini Review: On the elasticity of wheat gluten. Journal of Cereal Science 29, 103-107.

Berot, S., Gautier, S., Nicolas, M., Godon, B., Popineau, Y., 1994. Pilot scale preparation of wheat gluten protein fraction I-Influence of process parameters on their protein composition. International Journal of Food Science and Technology 29, 489-502.

Bietz, J.A. 1985. High performance liquid chromatography: How proteins look in cereals. Cereal Chemistry 62, 210-212.

Bietz, J.A., 1983. Separation of cereal proteins by reversed-phase high-performance liquid chromatography. Journal of Chromatography 255, 219-238.

Bietz, J.A., Cobb, L.A., 1985. Improved procedures for rapid wheat varietal identification by reversed-phase high-performance liquid chromatography of gliadin. Cereal Chemistry 62, 332-339.

Bietz, J.A., Simpson, D.G., 1992. Electrophoresis and chromatography of wheat proteins: available methods, and procedures for statistical evaluation of the data. Journal of Chromatography A 624, 53-80.

Bietz, J.A., Wall, J.S., 1975. The effect of various extractants on the subunits composition and associations of wheat glutenin. Cereal Chemistry 52, 145-155.

Biswas, S.C., Dubreil, L., Marion, D., 2001. Interfacial behaviour of wheat puroindolines: Monolayers of puroindolines at the air-water interface. Colloid & Polymer Science 279, 607-614.

Bitman, J., Wood, D.L., 1982. An improved copper reagent for quantitative densitometric thin-layer chromatography of lipids. Journal of Liquid Chromatography 5, 1155. 82.

Bloch, H.A., Darlington, H.F., Shewry, P.R., 2001. In vitro binding of puroindolines to wheat starch granules. Cereal Chemistry 78, 74-78.

Blochet, J.E., Chevalier, C., Forest, E., Pebay-Peyroula, E., Gautier, M.F., Joudrier, P., Pézolet, M., Marion, D., 1993. Complete amino acid sequence of puroindoline, a new basic protein with a unique tryptophan-rich domain, isolated from wheat

endosperm by Triton X-114 phase partitioning. FEBS Letters 329, 336-340.

Blochet, J.E., Kaboulou, A., Compoint, J.P., Marion, D., 1991. Amphiphilic proteins from wheat flour: Specific extraction, structure and lipid binding properties. In: Bushuk, W., Tkachuk, R. (Eds.), Gluten Protein 1990. American Association Cereal Chemists, St. Paul, Minnesota, pp. 314-325.

Boland, M., Brester, G.W., Taylor, M.R. 2005. Global and U.S. wheat gluten industries: structure, competition, and trade. Briefing No. 76. Agriculture Marketing Policy Centre, Bozeman, MT.

Bradshaw, T.P., 1998. HPLC Techniques for Analysis of Peptides and Proteins. A User's Guide. Phenomenex., California.

Branlard, G., Amiour, N., Igrejas, G., Gaborit, T., Herbette, S., Dardevet, M., Marion, D., 2003. Diversity of puroindolines as revealed by two-dimensional electrophoresis. Proteomics 3, 168-174.

Brooker, B.E., 1996. The role of fat in the stabilisation of gas cells in bread dough. Journal of Cereal Science 24, 187-198.

Burnouf, T., Bietz, J. A., 1989. Rapid purification of wheat glutenin for reversed phase high performance liquid chromatography: Comparison of dimethyl sulfoxide with traditional solvents. Cereal Chemistry 66, 121-127.

Bushuk, W., 1985. Protein-lipid and protein-carbohydrate interactions in flour-water mixtures . In: Blanshard, J.M.V., Frazier, P.J., Galliard, T. (Eds.), Chemistry and Physics of Baking. The Royal Society of Chemistry, London, UK, pp. 147-154.

Bushuk, W., 1998. Interactions in wheat doughs. In: Hamer, R.J., Hoseney, R.C. (Eds.), Interactions: The Key to Cereal Quality. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 1-15.

Bushuk, W., Zillman, R.R., 1978. Wheat cultivar identification by gliadin electrophoregrams. I. Apparatus, method and nomenclature. Canadian Journal of Plant Science 58, 505-515.

Byers, M., Miflin, B.J., Smith, S.J., 1983. A quantitative comparison of the extraction of protein fractions from wheat grain by different solvents and of the polypeptide and amino acid composition of the alcohol-soluble proteins. Journal of the Science of Food and Agriculture 34, 447-462.

Cargill, 2006. Cargill to Start Production of Vital Wheat Gluten in Russia. [On-line Internet]. Available: http://www.cargill.com/news_releases/060404_vitalwheatgluten.htm. Accessed 06 September 2006

Carr, N.O., Daniels, N.W.R., Frazier, P.J., 1992. Lipid interactions in Breadmaking. Critical Reviews in food Science and Nutrition 31, 237-258.

Castagnaro, A., García-Olmedo, F., 1994. A fatty-acid-binding protein from wheat kernels. FEBS Letters 349, 117-119.

Charlesworth, J.M., 1987. Evaporative analyzer as a mass detector for liquid chromatography. Analytical Chemistry 50, 1414-1420.

Charvolin, D., Douliez, J.P., Marion, D., Cohen-Addad, C., Pebay-Peyroula, E., 1999. The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. European Journal of Biochemistry 264, 562-568.

Chen, C. H., Bushuk, W., 1970. Nature of proteins in *Triticale* and its parental species. I. Solubility characteristics and amino acid composition of endosperm proteins. Canadian Journal of Plant Science 50, 9-14.

Christie, W.W., 1987. High-performance Liquid Chromatography and Lipids - A Practical Guide. Pergamon Press, Oxford, England, pp. 8-41.

Christie, W.W., Urwin, R.A., 1995. Separation of lipid classes from plant tissues by HPLC on chemically bonded stationary phases. Journal of High Resolution Chromatography 18, 97-100.

Chung, O.K., 1986. Lipid-protein interactions in wheat flour, dough, gluten, and protein fractions. Cereal Foods World 31, 242-256.

Chung, O.K., Ohm, J., 2000. Cereal lipids. In: Kulp, K., Ponte, J.G.Jr. (Eds), Handbook of Cereal Science and Technology. Marcel Dekker, New York, pp. 417-477.

Chung, O.K., Tsen, C.C., 1975a. Changes in lipid binding and distribution during dough mixing. Cereal Chemistry 52, 533-548.

Chung, O.K., Tsen, C.C., 1975b. Changes in lipid binding and protein extractability during dough mixing in presence of surfactants. Cereal Chemistry 52, 549-560.

Chung, O.K., Tsen, C.C., 1975c. Distribution of lipids in acid-soluble protein components as affected by dough-mixing and surfactants. Cereal Chemistry 52, 823-833.

Clayton, T.A., MacMurray, T.A., Morrison, W.R., 1970. Identification of wheat flour lipids by thin-layer chromatography. Journal of Chromatography 47, 277-281.

Clements, R.L., Donelson, J.R., 1981. Functionality of specific flour lipids in cookies. Cereal Chemistry 58, 204-206.

Colborne, A.J., Laidman, D.L., 1975. The extraction and analysis of wheat phospholipids. Phytochemistry 14, 2639-2545.

Cole, E.W., Mecham, D.K., Pence, J.W., 1960. Effect of flour lipids and some lipid derivatives on cookie-baking characteristics of lipid-free flours. Cereal Chemistry 37, 109-120.

Conforti, F.D., Harris, C.H., Rinehart, J.T., 1993. High-performance liquid chromatographic analysis of wheat flour lipids using an evaporative light scattering detector. Journal of Chromatography A 645, 83-88.

Cornec, M., Popineau, Y., Lefebvre, J., 1994. Characterisation of gluten subfractions by SE-HPLC and dynamic rheological analysis in shear. Journal of Cereal Science 19, 131-139.

Cornell, H.J., Hoveling, A.W., 1998a. The milling of wheat. In: Wheat : Chemistry and Utilization. Technomic Publishing Company Inc., Lancaster, Pennsylvania, pp: 43-78.

Cornell, H.J., Hoveling, A.W., 1998b. The wheat kernel. In: Wheat: Chemistry and Utilization . Technomic Publishing Company Inc, Lancaster, Pennsylvania, pp. 1-42.

Cornell, H.J., Hoveling, A.W., 1998c. Wheat proteins. In: Wheat : Chemistry and Utilization. Technomic Publishing Company Inc., Lancaster, Pennsylvania, pp. 327-374.

Cornell, H.J., Hoveling, A.W., 1998d. Wheat: Chemistry and Utilization. Technomic Publishing Company Inc., Lancaster, Pennsylvania, pp. 255-325.

Dachkevitch, T., Autran, J.C., 1989. Prediction of baking quality of bread wheats in breeding programs by size-exclusion high-performance liquid chromatography. Cereal Chemistry 66, 448-456.

Damodaran, S., 1996. Amino acids, peptides, and proteins. In: Fennema, O.R. (Eds.), Food Chemistry. Marcel Dekker, NewYork, pp. 321-429.

Daniels, N.W.R., Frazier, P.J., Wood, P.S., 1971. Flour lipids and dough development. Baker's Digest 45 August, 20-28.

Daniels, N.W.R., Richmond, J.W., Rusel Eggitt, O.W., Coppock, J.B., 1969. Studies on the lipids of flour. IV. Factors affecting lipid binding in breadmaking. Journal of the Science of Food and Agriculture 20, 129-136.

Davies, R.J., Daniels, N.W.R., Greenshield, R.N., 1969. An improved method of adjusting moisture in studies on lipid binding. Journal of Food Technology 4, 117-123.

Day, L., 2004. Lipid chemistry. In: Wrigley, C.W., Corke, H., Walker, C.E. (Eds.), Encyclopedia of Grain Science, Volume 2. Elsevier Academic Press, Oxford, pp. 157-166.

Day, L., Augustin, M.A., Batey, I.L., Wrigley C.W., 2006. Wheat-gluten uses and industry needs. Trends in Food Science and Technology 17, 82-90.

Day, L., Bhandari, D.G., Greenwell, P., Schofield, J.D., 2002. Biochemical studies pf puroindoline proteins in relation to the endosperm texture of hexaploid wheat: Water Bushuk student award paper. In: Ng, P.K.W., Wrigley, C. W. Wheat Quality Elucidation: The Bushuk Legacy. 234-243. St. Paul, Minnesota, American Association of Cereal Chemists.

Day, L., Greenwell, P., Lock, S., Brown, H., 1999. Analysis of wheat flour proteins related to grain hardness using capillary electrophoresis. Journal of

Chromatography A 836, 147-152.

Day, L., Greenwell, P., Lock, S., Brown, H., 1999. Analysis of wheat flour proteins related to grain hardness using capillary electrophoresis. Journal of Chromatography A 836, 147-152.

Day, L., Schofield, J.D., 2001. Biochemical studies of puroindoline proteins in relation to the endosperm texture of wheat. Aspect of Applied Biology 64, 63-68.

Dill, D.B., 1925.The composition of crude gluten. Cereal Chemistry 2, 1-11.

Dougherty, D.A., Zeece, M.G., Wehling, R.L., Partridge, J.E., 1989. High-Resolution two-dimensional electrophoresis of wheat proteins. Journal of Chromatography A 480, 359-369.

Douliez, J.P., Jegou, S., Pato, C., Larre, C., Molle, D., Marion, D., 2001a. Identification of a new form of lipid transfer protein (LTP1) in wheat seeds. Journal of Agricultural and Food Chemistry 49, 1805-1808.

Douliez, J.P., Jegou, S., Pato, C., Molle, D., Tran, V., Marion, D., 2001b. Binding of two mono-acylated lipid monomers by the barley lipid transfer protein, LTP1, as viewed by fluorescence, isothermal titration calorimetry and molecular modelling. European Journal of Biochemistry 268, 384-388.

Douliez, J.P., Michon, T., Elmorjani, K., Marion, D., 2000a. Mini review: Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. Journal of Cereal Science 32, 1-20.

Douliez, J.P., Michon, T., Marion, D., 2000b. Steady-state tyrosine fluorescence to study the lipid-binding properties of a wheat non-specific lipid-transfer protein (nsLTP1). Biochimica et Biophysica Acta 1467, 65-72.

Douliez, J.P., Pato, C., Rabesona, H., Molle, D., Marion, D., 2001c. Disulfide bond assignment, lipid transfer activity and secondary structure of a 7-Kda plant lipid transfer protein, LTP2. European Journal of Biochemistry 268, 1400-1403.

Du Cros, D.L., Wrigley, C.W., 1979. Improved electrophoretic methods for identifying cereal varieties. Journal of the Science of Food and Agriculture 30, 785-794.

Dubreil, L., Biswas, S.C., Marion, D., 2002. Localization of puroindoline-a and lipids in bread dough using confocal scanning laser microscopy. Journal of Agricultural and Food Chemistry 50(21), 6078-6085.

Dubreil, L., Compoint, J.P., Marion, D., 1997. Interaction of puroindolines with wheat flour polar lipids determines their foaming properties. Journal of Agricultural and Food Chemistry 45(1), 108-116.

DuPont, F.M., Chan, R., Lopez, R., Vensel, W.H., 2005. Sequential extraction and quantitative recovery of gliadins, glutenins, and other proteins from sample samples of wheat flour. Journal of Agricultural and Food Chemistry 53, 1575-1584.

DuPont, F.M., Vensel, W.H., Chan, R., Kasarda, D.D., 2000. Characterization of the 1B-type w-gliadins from *Triticum aestivum* cultivar butte. Cereal Chemistry 77, 607-614.

Dworschak, R.G., Ens, W., Standing, K.G., Preston, K.R., Marchylo, B.A., Nightingale, M.J., Stevenson, S.G., Hatcher, D.W., 1998. Analysis of wheat gluten proteins by natrix-assisted laser desorption/ionization mass spectrometry. Journal of Mass Spectrometry 33, 429-435.

Fagan, P., Wijesundera, C., Watkins, P., 2004. Liquid chromatographic analysis of milk phospholipids with on-line pre-concentration. Journal of Chromatography A 1054, 241-249.

FAIR-CT96-1979, 2000. Wheat Gluten as Biopolymer for the Production of Renewable and Biodegradable Materials. [On-line Internet]. Available: http://www.biomatnet.org/secure/Fair/R1979.htm, CPL Press. Accessed 06 September 2006.

Farrer, K.T.H., 1988. Food Technology. Technology in Australia 1788-1988. Australian Science and Technology Heritage Centre, Melbourne, pp. 94-98.

Frazier, P.J, Daniels, N.W.R., Eggitt, P.W.R., 1981. Lipid-Protein interactions during dough development. Journal of the Science of Food and Agriculture 32, 877-897.

Frazier, P.J., 1983. Lipid-protein interactions during dough development. In: Barnes, P.J. (Eds.), Lipids in cereal technology. Academic Press, London, pp. 189-212.

Fu, B.X., Kovacs, M.I.P., 1999. Research note: Rapid single-step procedure for isolating total glutenin proteins of wheat flour. Journal of Cereal Science 29, 113-116.

Fu, B.X., Sapirstein, H.D., 1996. Procedure for isolating monomeric proteins and polymeric glutenin of wheat flour. Cereal Chemistry 73, 143-152.

Gan, Z., Ellis, P.R., Schofield, J.D., 1995. Gas cell stabilization and gas retention in wheat bread dough. Journal of Cereal Science 21, 215-230.

Georgopoulos, T., Larsson, H., Eliasson, A.C., 2006. Influence of native lipids on the rheological properties of wheat flour dough and gluten. Journal of Texture Studies 37, 49-62.

Grace, G., 1988. Preparation of vital wheat gluten. In: World Congress of Vegetable Protein Utilization in Human Food and Animal Feedstuffs, Singapore.

Graybosch, R. A., Morris, R., 1990. An improved SDS-PAGE method for the analysis of wheat endosperm storage proteins. Journal of Cereal Science 11, 201-212.

Greenspan, P., Mao, F-W., Ryu, B-H., Gutman, R.L., 1995. Advances in agarose gel electrophoresis of serum lipoproteins. Journal of Chromatography 698, 333-339.

Guilbert, S., Gontard, N., Morel, M.H., Chalier, P., Micard, V., Redl, 2002. A

formulation of properties of wheat gluten films and coating. In: Gennadios, A. (Ed.), Protein-Based Films and Coating. CRC Press, Boca Raton, Florida, pp. 69-122.

Han, G.W., Lee, J.Y., Song, H.K., Chang, C., Min, K., Moon, J., Shin, D.H., Kopka, M.L., Sawaya, M.R., Yuan, H.S., Kim, T.D., Choe, J., Lim, D., Moon, H.J., Suh, S.W. 2001. Structural basis of non-specific lipid binding in maize lipid-transfer protein complexes revealed by high-resolution x-ray crystallography. Journal of Molecular Biology 308, 263-278.

Hargreaves, J., Le Meste, M., Popineau, Y., 1994. ESR studies of gluten-lipid systems. Journal of Cereal Science 19, 107-113.

Harwood, J.L., 1994. Lipid Structure. In: Gunstone, F.D., Harwood, J.L., Padley, F.B. (Eds.), The Lipid Handbook. Chapman & Hall, London, UK, pp. 21-33.

Helenius, A., Simons, K., 1975. Solubilization of membranes by detergents. Biochimica et Biophysica Acta 415, 29-79.

Holcomb, R.B., 2000. Fact Sheet 559: Overview of the Domestic Wheat Gluten Industry. Oklahoma State University, Stillwater, Okalahoma, pp: 1-4.

Hoseney, R.C., 1998a. Gluten Proteins. In: Principles of Cereal Science and Technology, second ed. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 197-211.

Hoseney, R.C., 1998b. Proteins of cereals. In: Principles of Cereal Science and Technology, second ed. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 65-80.

Hoseney, R.C., Finney, K.F., Pomeranz, Y., 1970a. Functional (breadmaking) and biochemical properties of wheat flour components. VI. Gliadin-lipid-glutenin interaction in wheat gluten. Cereal Chemistry 47, 135-140.

Hoseney, R.C., Pomeranz, Y., Finney, K.F., 1970b. Functional (breadmaking) and biochemical properties of wheat flour components. VII. Petroleum ether-soluble lipoproteins of wheat flour. Cereal Chemistry 47, 153-160.

Huang, D.Y., Khan, K., 1998. Research note: A modified SDS-PAGE procedure to separate high molecular weight glutenin subunits 2 and 2*. Journal of Cereal Science 27, 237-239.

Huebner, F.R., Bietz, J.A., 1985. Detection of quality differences among wheats by high-performance liquid chromatography. Journal of Chromatography 327, 333-342.

Islam, N., Tsujimoto, H., Hirano, H., 2003. Proteome analysis of diploid, tetraploid and hexaploid wheat: Towards understanding genome interaction in protein expression. Proteomics 3, 549-557.

Jackson, E.A., Holt, L.M., Payne, P.E., 83. Characterisation of high molecular weight gliadin and low molecular weigh glutenin subunits of wheat endosperm by two-

dimensional electrophoresis and the chromosomal location of their controlling genes. Theoretical and Applied Genetics 66, 29-37.

JCS. 2006. Journal of Cereal Science – Guide for Authors. [On-line Internet]. Available: http://www.elsevier.com/wps/find/journaldescription.cws_home/622859/authorinstruc… Access 28 July 2006.

Jones, B.L., Morris, C.F., Bekes, F., Wrigley, C. W. 2006. Proteins that complement the roles of gliadin and glutenin. In: Wrigley, C., Bekes, F., Bushuk, W. (Eds.), Gliadin and Glutenin: The Unique Balance of Wheat Quality. AACC International, St. Paul, Minnesota, pp. 413-446.

Jones, R.W., Taylor, N.W., Senti, F.R., 1959. Electrophoresis and fractionation of wheat gluten. Archives of Biochemistry and Biophysics 84, 363-376.

Kaldy, M.S., Kereliuk, G.R., Kozub, G.C., 1993. Influence of gluten components and flour lipids on soft white wheat quality. Cereal Chemistry 70, 77-80.

Kasarda, D.D., Adalsteins, A.E., Laird, N.F., 1987. Gamma-gliadins with alpha-type structure coded on chromosome 6B of the wheat (*Triticum aestivum* L.) cultivar 'Chinese Spring". In: Lasztity, R., Békés, F. (Eds.) Proceedings of the Third International Workshop on Gluten Proteins. World Scientific Publishing, Singapore, pp. 20-29.

Kasarda, D.D., Woodard, K.M., Adalsteins, A.E., 1998. Resolution of high molecular weight glutenin subunits by a new SDS-PAGE system incorporating a neutral pH buffer. Cereal Chemistry 75, 70-71.

Keller, R.C.A., Orsel, R., Hamer, R.J., 1997. Competitive adsorption behaviour of wheat flour components and emulsifiers at an air-water interface. Journal of Cereal Science 25(2), 175-183.

Khan, K., Hamada, A.S., Patek, J., 1985. Polyacrylamide gel electrophoresis for wheat variety identification: Effect of variables on gel properties. Cereal Chemistry 62, 310-313.

Khan, K., Huckle, L., 1992. Use of multistacking gels in sodium dodecyl sulfate-polyacrylamide gel electrophoresis to reveal polydispersity, aggregation, and disaggregation of the glutenin protein fraction . Cereal Chemistry 69, 686-688.

Khan, K., McDonald, C.E., Banasik, O.J., 1983. Polyacrylamide gel electrophoresis of gliadin proteins for wheat variety identification - procedural modification and observations. Cereal Chemistry 60, 178-181.

Khan, K., Nygard, G., Pogna, N.E., Redaelli, R., Ng, P.K.W., Fido, R.J., Shewry, P.R., 2003. Electrophoresis of wheat gluten proteins. In: Shewry, P.R., Lookhart, G.L. (Eds.) Wheat Gluten Protein Analysis. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 31-59.

Khelifi, D., Branlard, G., 1991. A new two-step electrophoresis method for analysing gliadin polypeptides and high and low molecular weight subunits of glutenin of

wheat. Journal of Cereal Science 13, 41-47.

Kissell, L.T., Pomeranz, Y., Yamazaki, W.T., 1971. Effects of flour lipids on cookies quality. Cereal Chemistry 48, 655-662.

Koehler, P., 2003. Amino acid and protein sequence analysis. In: Shewry, P.R., Lookhart, G.L. (Eds.), Wheat Gluten Protein Analysis. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 137-158.

Konopka, I., Czaplicki, S., Rotkiewicz, D., 2006. Differences in content and composition of free lipids and carotenoids in flour of spring and winter wheat cultivated in Poland. Food Chemistry 95, 290-300.

Kooijman, M., Orsel, R., Hessing, M., Hamer, R.J., Bekkers, A.C.A.P.A., 1997. Spectroscopic characterisation of the lipid-binding properties of wheat puroindolines. Journal of Cereal Science 26, 145-159.

Kruger, J.E., Marchylo, B.A., Hatcher, D., 1988. Preliminary assessment of sequential extraction scheme for evaluating quality by reversed-phase high-performance liquid chromatography and electrophoretic analysis of gliadins and glutenins. Cereal Chemistry 65, 208-214.

Larré, C., Nicolas, Y., Desserme, C., Courcoux, P., Popineau, Y., 1997. Preparative separation of high and low molecular weight subunits of glutenin from wheat. Journal of Cereal Science 25, 143-150.

Larroque, O.R., Gianibelli, M.C., Gomez Sanchez, M., MacRitchie, F., 2000. Procedure for obtaining stable protein extracts of cereal flour and whole meal fo size-exclusion HPLC analysis. Cereal Chemistry 77, 448-450.

Larson, K. and Quinn, P.J., 1994. Physical properties: Structural and physical characteristics. In: Gunstone, F.D., Harwood, J.L., Padley, F.B. (Eds.), The Lipid Handbook. Chapman and Hall, London, UK, pp. 401-457.

Larsson, K., 1985. Functionality of wheat lipids in relation to gluten gel formation. In: Blanshard, J.M.V., Frazier, P.J., Galliard, Y. (Eds.), Chemistry and Physics of Baking. J. W. Arrowsmith Ltd, Bristol, pp. 62-74.

Lasztity, R., 1996. Wheat protein. In: The Chemistry of Cereal Proteins, second ed. CRC Press, Boca Raton, Florida, pp. 19-138.

Lew, E.J-L., Kuzmicky, D.D., Kasarda, D.D., 1992. Characterization of low molecular weight glutenin subunits by reverse-phase high performance liquid chromatography, sodium dodecyl sulfate-polyacrylamide gel electrohporesis, and N-terminal amino acid sequencing. Cereal Chemistry 69, 508-515.

Li, W., Dobraszczyk, B.J., Wilde, P.J., 2004. Surface properties and locations of gluten proteins and lipids revealed using confocal scanning laser microscopy in bread dough. Journal of Cereal Science 39, 403-411.

Lillemo, M., Morris, C.F., 2000. A leucine to proline mutation in puroindoline-b is frequently present in hard wheats from Northern Europe. Theoretical and

Applied Genetics 100, 1100-1107.

Lookhart, G., Bean, S., 1995. A fast method for wheat cultivar differentiation using capillary zone electrophoresis. Cereal Chemistry 72, 42-47.

Lookhart, G., Bean, S., 2000. Cereal Proteins: Composition of their major fractions and methods for identification. In: Kulp, K., Ponte, J.G.Jr. (Eds), Handbook of Cereal Science and Technology. Marcel Dekker, NewYork, pp 363-383.

Lookhart, G.L., Bean, S.R., 1996. Improvements in cereal protein separations by capillary electrophoresis: Resolution and reproducibility. Cereal Chemistry 73, 81-87.

Lookhart, G.L., Bean, S.R., Bietz, J.A., 2003. HPLC of gluten monomeric proteins. In: Shewry, P.R., Lookhart, G.L. Wheat Gluten Protein Analysis. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 61-89.

Lookhart, G.L., Cooper, D.B., Jones, B.L., 1985. Effect of temperature and alternate lactate buffer systems on resolution of wheat gliadin proteins by polyacrylamide gel electrophoresis. Cereal Chemistry 62, 19-22.

Lookhart, G.L., Jones, B.L., Hall, S.B., Finney, K.F., 1982. An improved method for standardizing polyacrylamide gel electrophoresis of wheat gliadin proteins. Cereal Chemistry 59, 178-181.

Lookhart, G.L., Menkovska, M., Pomeranz, Y., 1989. Polyacrylamide gel electrophoresis and high-performance liquid chromotography patterns of gliadins from wheat sections and milled and air-classified fractions. Cereal Chemistry 66, 256-262.

MacMurray, T.A., Morrison, W.R., 1970. Composition of wheat-flour lipids. Journal of the Science of Food and Agriculture 21, 520-528.

MacRitchie, F., 1983. Role of lipids in baking. In: Barnes, P.J. (Eds.), Lipids in Cereal Technology. Academic Press, London, pp. 165-188.

MacRitchie, F., 1985. Studies of the methodology for fractionation and reconstitution of wheat flours. Journal of Cereal Science 3, 221-230.

MacRitchie, F., 1987. Evaluation of contributions from wheat protein fractions to dough mixing and breadmaking. Journal of Cereal Science 6, 259-268.

MacRitchie, F., Gras, P.W., 1973. The role of flour lipids in baking. Cereal Chemistry 50, 292-302.

MacRitchie, F., Kasarda, D.D., Kuzmicky, D.D., 1991. Characterization of wheat protein fractions differing in contributions to breadmaking quality. Cereal Chemistry 68, 122-130.

Marchal, R., Marchal, D.L., Lallement, A., Jeandet, P., 2002.Wheat gluten used as a clarifying agent of red wine. Journal of Agriculture and Food Chemistry 50, 177-184.

Marchylo, B., Kruger, J.E., Irvine, G.N., 1976. Alpha-amylase from immature hard red spring wheat. I. Purification and some chemical and physical properties. Cereal Chemistry 53, 157-173.

Marchylo, B.A., Kruger, J.E., Hatcher, D.W., 1989. Quantitative reversed-phase high-performance liquid chromatographic analysis of wheat storage protein as a potential quality prediction tool. Journal of Cereal Science 9, 113-130.

Marion, D., Clark, D.C., 1995. Wheat Lipids and Lipid-Binding Proteins: Structure and Function. In: Schofield, J.D. (Eds), Wheat Structure, Biochemistry and Functionality. The Royal Society of Chemistry, Cambridge, UK, pp. 245-260.

Marion, D., Dubreil, L., Douliez, J.P., 2003. Functionality of lipids and lipid-protein interactions in cereal-derived food products. Ocl-Oleagineux Corps Gras Lipides 10, 47-56.

Marion, D., Dubriel, L., Wilde, P.J., Clark, D.C., 1998. Lipids, lipid-protein interactions and the quality of baked cereal products. In: Hamer, R.J., Hoseney, R.C. (Eds.), Interactions: The Keys to Cereal Quality. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 131-167.

Marion, D., Roux, C.L., Akoka, S., Tellier, C., Gallant, D., 1987. Lipid-protein interactions in wheat gluten: a phosphorus nuclear magnetic resonance spectroscopy and freeze-fracture electron microscopy study. Journal of Cereal Science 5, 101-115.

Masci, S.M., Porceddu, E., Colaprico, G., Lafiandra, D., 1991. Comparison of the B and D subunits of glutenin encoded at the *Glu-D3* locus in two biotypes of the common wheat cultivar Newton with different technological characteristics. Journal of Cereal Science 14, 35-46.

McCormack, G., Panozzo, J., Bekes, F., MacRitchie, F., 1991. Contributions to breadmaking of inherent variations in lipid content and composition of wheat cultivars. I. Results of survey. Journal of Cereal Science 13, 255-261.

Mecham, D.K., Mohammed, A., 1955. Extraction of lipids from wheat products. Cereal Chemistry 32, 405-415.

Meredith, P., Sammons, H.G., Frazer, A.C., 1960. Examination of wheat gluten by partial solubility methods. I. Partition by organic solvent. Journal of the Science of Food and Agriculture 11, 320-328.

Mohamed, A., Gordon, S.H., Harry-O'Kuru, R., Palmquist, D.E., 2005. Phospholipids and wheat gluten blends: interaction and kinetics. Journal of Cereal Science 41, 259-265.

Moreau, R.A., 1994. Quantitative analysis of lipids by HPLC with a flame-ionization detector or an evaporative light-scattering detector. In: Shibamoto, T. (Ed.), Lipid Chromatographic Analysis. Chromatographic Science Serie. Volume 65. Marcel Dekker, New York, pp. 251-272.

Morel, M.H., 1994. Acid-polyacrylamide gel electrophoresis of wheat glutenin: A new

tool for the separation of high and low molecular weight subunits. Cereal Chemistry 71, 238-242.

Morris, C.F., Greenblatt, G.A., Malkawi, H.I., 1992. Enhanced electrophoretic detection and isolation of friabilin, a starch granule protein. Cereal Chemistry 69, 467-468.

Morrison, R., 1976. Lipids in flour, dough and bread. The Bakers Digest, 29-36, 47-48.

Morrison, W.R, Mann, D.L., Soon, W., Coventry, A.M., 1975. Selective extraction and quantitative analysis of non-starch and starch lipids from wheat flour. Journal of the Science of Food and Agriculture 26, 507-521.

Morrison, W.R., 1968. The distribution of phospholipids in some mammalian milks. Lipids 3, 101-103.

Morrison, W.R., 1978. Cereal lipids. In: Pomeranz, Y. (Ed.), Advances in Cereal Science and Technology, Volume II. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 221-348.

Morrison, W.R., 1983. Acyl lipids in cereals. In: Barnes, P.J. (Ed.), Lipid in Cereal Technology. Academic Press, London, pp. 11-32.

Morrison, W.R., 1988. Lipids in cereal starches: A Review. Journal of Cereal Science 8, 1-15.

Morrison, W.R., 1988. Lipids. In: Pomeranz, Y. (Ed.), Wheat Chemistry and Technology. American Association of Cereal Chemistry, St. Paul, Minnesota, pp. 373-439.

Morrison, W.R., Coventry, A.M., 1985. Extraction of lipids from cereal starches with hot aqueous alcohols. Starch 37, 83-87.

Morrison, W.R., Tan, L.S., Hargin, K.D., 1980. Methods for the quantitative analysis of lipids in cereal grains and similar tissue. Journal of the Science of Food and Agriculture 31, 329-340.

Mugford, D.C., Batey, I.L., 1989. Composition of Australian flour mill products from bakers' wheat grist. Food Australia 41, 554-559.

Néron, S., Amrani, F.E., Potus, J., Nicolas, J., 2004. Separation and quantification by high-performance liquid chromatography with light scattering detection of the main wheat flour phospholipids during dough mixing in the presence of phospholipase. Journal of Chromatography A 1047, 77-83.

Nicolas, Y., Larré, C., Popineau, Y., 1997. A method for the isolation of high M_r subunits of wheat glutenin. Journal of Cereal Science 25, 151-154.

Nicolas, Y., Martinant, J-P., Denery-Papini, S., Popineau, Y., 1998. Analysis of wheat storage proteins by exhaustive sequential extraction followed by RP-HPLC and nitrogen determination. Journal of the Science of Food and Agriculture 77, 96-102.

Nordbäck, J., Lundberg, E., Christie, W.W., 1998. Separation of lipid classes from marine particulate material by HPLC on a polyvinyl alcohol-bonded stationary phase using dual-channel evaporative light-scattering detection. Marine Chemistry 60, 165-175.

Ohm, J.B., Chung, O.K., 2000. NIR transmittance estimation of free lipid content and its glycolipid and digalactosyldiglyceride contents using wheat flour lipid extracts. Cereal Chemistry 77, 556-559.

Ohm, J.B., Chung, O.K., 2002. Relationships of free lipids with quality factors in hard winter wheat flour. Cereal Chemistry 79, 274-278.

Olcott, H.S., Mecham, D.K., 1947. Characterization of wheat gluten. I. Protein-lipid complex formation during doughing of flours. Lipoprotein nature of the glutenin fraction. Cereal Chemistry 24, 407-414.

Örnebro, J., Nylander, T., Eliasson, A.C. 2000. Critical review - interfacial behaviour of wheat proteins. Journal of Cereal Science 31, 195-221.

Osborne, T. B., 1924. Vegetable Proteins. Longmans Green, London, UK, pp. 154.

Panozzo, J.F., O'Brien, L.O., MacRitchie, F., Bekes, F, 1990. Baking quality of australian wheat cultivars varying in their free lipid composition. Journal of Cereal Science 11, 51-57.

Papantoniou, E., Hammond, E.W., Scriven, F., Gordon, M.H., Schofield, J.D., 2001. Isolation of polar lipid classes from wheat flour extracts by preparative high-performance liquid chromatography. Cereal Chemistry 78, 663-665.

Papantoniou, E., Hammond, E.W., Scriven, F., Gordon, M.H., Schofield J.D., 2004. Effects of endogenous flour lipids on the quality of short-dough biscuits. Journal of The Science of Food and Agriculture 84, 1371-1380.

Papantoniou, E., Hammond, E.W., Tsiami, A.A., Scriven, F., Gordon, M.H., Schofield, J.D., 2003. Effects of endogenous flour lipids on the quality of semisweet biscuits. Journal of Agricultural and Food Chemistry 51, 1057-1063.

Paternotte, T.A., Orsel, R., Hamer, R.J., 1994. Dynamic interfacial behaviour of gliadin-diacylgalactosylglycerol (MGDG) films: Possible implications for gas-cell stability in wheat flour doughs. Journal of Cereal Science 19, 123-129.

Payne, P.I., Corfield, K.G., Blackman, J.A., 1979. Identification of a high-molecular-weight subunit of glutenin whose presence correlates with bread-making quality in wheats of related pedigree. Theoretical and Applied Genetics 55, 153-159.

Payne, P.I., Holt, L.M., Jarvis, M.G., Jackson, E.A., 1985. Two-dimensional fractionation of the endosperm proteins of bread wheat (*Triticum aestivum*): Biochemical and genetic studies. Cereal Chemistry 62, 319-326.

Payne, P.I., Holt, L.M., Law, C.N., 1981. Structural and genetical studies on the high-molecular-weight subunits of wheat glutenin. Theoretical and Applied Genetics 60, 229-236.

Pezolet, M., Bonenfant, S., Dousseau, S., Popineau, Y., 1992. Conformation of wheat gluten proteins: Comparison between functional and solution states as determined by infrared spectroscopy. FEBS Letters 299, 247-250.

Pomeranz, Y., 1987. Modern Cereal Science and Technology. VCH Publishers, New York, pp. 165-200.

Pomeranz, Y., Chung, O.K., 1965. The lipid composition of a single wheat kernel and its structural parts. Journal of Chromatography A 19, 540-550.

Pomeranz, Y., Tao, R.P., Hoseney, R.C., Shogren, M.D., Finney, K.F., 1968. Evaluation of factors affecting lipid binding in wheat flours. Journal of Agricultural and Food Chemistry 16, 974-978.

Ponte, J.G.Jr., De Stefanis, V.A., Cotton, R.H., 1967. Studies of gluten lipids. I. Distribution of lipids in gluten fractions separated by solubility in 70% ethanol. Cereal Chemistry 44, 427-435.

Ponte, J.G.Jr., Dogan, I.S., Kulp, K., 2000. Special food ingredients from cereals. In: Kulp K., Ponte, J.G.Jr. (Eds.), Handbook of Cereal Science and Technology. Marcel Dekker, NewYork, pp. 755-775.

Posner, E.S., Hibbs, A.N., 1997. Wheat flour milling. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 72.

Preston, K.R., Stevenson, S.G., 2003. Size exclusion chromatography and flow field-flow fractionation of wheat proteins. In: Shewry, P.R. and Lookhart, G.L. Wheat Gluten Protein Analysis. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 115-136.

Prieto, J.A., Weegels, P.L., Hamer, R.J., 1993. Functional properties of low M_r wheat proteins. I. Isolation, characterization and comparison with other reported low M_r wheat proteins. Journal of Cereal Science 17, 203-220.

Quimby, P.R.Jr., Birdall, J.L., Caesar, A.J. , Connick, W.J.Jr., Boyette, C.D., Caesar, T.C., Sands, D.C., 1994. Oil and absorbent coated granules containing encapsulated living organisms for controlling agricultural pests. US Patent 5,358,863.

Redman, D.G., Fisher, N., 1968. Fractionation and comparison of purothionin and globulin component of wheat. Journal of the Science of Food and Agriculture 19, 651-655.

Reichardt, C., 1988. Solvents and Solvent Effects in Organic Chemistry. VCH, Verlagsgesellschaft, Weinheim, pp. 407-437.

Ruibal-Mendieta, N.L., Delacroix, D.L., Meurens, M., 2002. A comparative analysis of free, bound and total lipid content on spelt and winter wheat wholemeal. Journal of Cereal Science 35, 337-342.

Sadouki, H., Cazalis, R., Azzout, B., 2005. Fractionation of Algerian common wheat proteins by HPLC and sodium dodecyl sulfate-polyacrylamide gel

electrophoresis; relationship with technological quality. LWT 38, 829-841.

Sahi, S.S., 2003. The interfacial properties of the aqueous phases of full recipe bread doughs. Journal of Cereal Science 37, 205-214.

Sanchez-Monge, R., Gomez, L., Garcia-Olmedo, F., Salcedo, G., 1986. A tetrameric inhibitor of insect α-amylase from barley. FEBS Letters 207, 105-109.

Sapirstein, H.D., Fu, B.X., 1998. Intercultivar variation in the quantity of monomeric proteins, soluble and insoluble glutenin, and residue protein in wheat flour and relationships to breadmaking quality. Cereal Chemistry 75, 500-507.

Shantha, N.C., Napolitano, G.E., 1998. Lipid analysis using thin-layer chromatography and the Iatroscan. In: Hamilton, R.J. (Ed.), Lipid Analysis in Oils and Fats. Blackie Academic & Professional, London, UK, pp. 1-33.

Shewry, P.R. Tatham, A.S., 1997. Disulphide bonds in wheat gluten proteins. Journal of Cereal Science 25, 207-227.

Shewry, P.R., 2003. Wheat Gluten Proteins. In: Shewry, P.R., Lookhart, G.L. Wheat Gluten Protein Analysis. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 1-17.

Shewry, P.R., Halford, N.G., Tatham, A.S. 1992. High molecular weight subunits of wheat glutenin. Journal of Cereal Science 15, 105-120.

Shewry, P.R., Popineau, Y., Lafiandra, D., Belton, P. 2001. Wheat glutenin subunits and dough elasticity: findings of the EUROWHEAT project. Trends in Food Science and Technology 11, 433-441.

Shin, D.H., Lee, J.Y., Qwang, K.Y., Kim, K.K., and Suh, S.W., 1995. High resolution crystal structure of the non specific lipid transfer protein from maize seedling. Structure 3, 189-199.

Singh, N.K., Donovan, G.R., Batey, I.L., MacRitchie, F., 1990. Use of sonication and size-exclusion high-performance liquid chromoatography in the study of wheat flour proteins. I. Dissolution of total proteins in the absence of reducing agents. Cereal Chemistry 67, 150-161.

Sinha, N.K., Yamamoto, H., Ng, P.K.W., 1997. Effects of flour chlorination on soft wheat gliadins analyzed by reversed-phase high-performance liquid chromatography, differential scanning calorimetry and fluorescence spectroscopy. Food Chemistry 59, 387-393.

Skylas, D.J., Mackintosh, J.A., Cordwell, S.J., Basseal, D.J., Walsh, B.J., Harry, J., Blumenthal, C., Copeland, L., Wrigley, C.W., Rathmell, W., 2000. Proteome approach to the characterisation of protein composition in the developing and mature wheat grain endosperm. Journal of Cereal Science 32, 169-188.

Skylas, D.J., Van Dyk, D., Wrigley, C.W., 2005. Review: Proteomics of wheat grain. Journal of Cereal Science 41, 165-179.

Sodano, P., Caille, A., Sy, D., Person, G., Marion, D., Ptak, M., 1997. ^{1}H NMR and fluorescence studies of the complexation of DMPG by wheat non-specific lipid transfer protein. Global fold of the complex. FEBS Letters 416, 130-134.

Spies, R.D., Kirleis, A.W., 1978. Effect of free flour lipids on cake-baking potential. Cereal Chemistry 55, 699-704.

Stevenson, S.G., Preston, K.R., 1996. Flow field-flow fractionation of wheat proteins. Journal of Cereal Science 23, 121-131.

Suchy, J., Lukow, O.M., Fu, B.X., 2003. Quantification of monomeric and polymeric wheat proteins and the relationship of protein fractions to wheat quality. Journal of the Science of Food and Agriculture 83, 1083-1090.

Takeda, K., 1994. Effects of various lipid fractions of wheat flour on expansion of sponge cake. Cereal Chemistry 71, 6-9.

Tatham, A.S., 1995. The structure of wheat proteins. In: Schofield, J.P. (Eds.), Wheat Structure: Biochemistry and Functionality. The Royal Society of Chemistry, Cambridge, UK, pp. 53-62.

Tatham, A.S., Shewry, P.R., 1995. The S-poor prolamins of wheat, barley and rye. Journal of Cereal Science 22, 1-16.

Tilley, K.A., Benjamin, R.E., Bagorogoza, K.E., Moses Okot-Kotber, B., Prakash, O., Kwen, H., 2001. Tyrosine cross-links: Molecular basis of gluten structure and function. Journal of Agricultural and Food Chemistry 49, 2627-2632.

Tsen, C.C., Levi, I., Hlynka, I., 1962. A rapid method for the extraction of lipids from wheat products. Cereal Chemistry 39, 195-203.

Tweeten, T.N., Wetzel, D.L., 1981. Physicochemical characterization of galactosyldiglycerides and their quantitation in wheat flour lipids by high-performance liquid chromatography. JAOCS, 664-672.

Van, D.E.R., Schueren, F.M.L., 2002. Chew candy or fruit chew comprises wheat gluten and maltodetrin as replacement for gelatin. European Patent 979711.

Wahlund, K.G., Gustavsson, M., MacRitchie, F., Nylander, T., Wannerberger, L., 1996. Size characterisation of wheat proteins, particularly glutenin, by asymmetrical flow field-flow fractionation. Journal of Cereal Science 23(2), 113-119.

Wasik, R.J., Bushuk, W., 1974. Studies of glutenin. V. Note on additional preparative methods. Cereal Chemistry 51, 112-118.

Wehrli, H.P., Pomeranz, Y., 1970. A note on the interaction between glycolipid and wheat flour macromolecules. Cereal Chemistry 47, 160-166.

Werner, W.E., Wiktorowicz, J.E., Kasarda, D.D., 1994. Wheat varietal identification by capillary electrophoresis of gliadins and high molecular weigh glutenin subunits. Cereal Chemistry 71, 397-402.

Wieser, H., Seimeier, W., Belitz, H-D., 1994. Quantitative determination of gliadins subgroups from different wheat cultivars. Journal of Cereal Science 19, 149-155.

Woychik, J.H., Boundy, A., Dimler, R.J., 1961. Starch gel electrophoresis of wheat gluten proteins with concentration urea. Archives of Biochemistry and Biophysics 94, 477-482.

Wrigley, C.W., 1970. Protein mapping by combined gel electrofocusing and electrophoresis: Application to the study of genotypic variations in wheat gliadins. Biochemical Genetics 4, 509-516.

Wrigley, C.W., Andrews, J.L., Bekes, F., Gras, P.W., Gupta, R.B., MacRitchie, F., Skerritt, J.H., 1998. Protein-protein interactions - Essential to dough rheology. In: Hamer, R.J., Hoseney, R.C. (Eds.), Interactions: The Keys to Cereal Quality. American Association of Cereal Chemists, St. Paul, Minnesota, pp. 17-46.

Wrigley, C.W., Bekes, F., Bushuk, W., 2006. Gluten: A balance of gliadin and glutenin. In: Wrigley, C.W., Bekes, F., Bushuk, W. (Eds.), Gliadin and Glutenin: The Unique Balance of Wheat Quality. AACC International, St. Paul, Minnesota, pp. 3-32.

Wrigley, C.W., Gore, P.J., Manusu, H.P., 1991. A rapid (<10 minute) electrophoresis method for identification of wheat varieties. Electrophoresis 12, 384-385.

Yepiz-Plascencia, G.M., Sotelo-Mundo, R., Vazquez-Moreno, L., Ziegler, R., Higuera-Ciapara, I., 1995. A non-sex-specific hemolymph lipoprotein from the white shrimp *Penaeus vannamei* Boone. Isolation and partial characterization. Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology 111B, 181-187.

Zawistowska, U., Bekes, F., Bushuk, W., 1985. Gluten proteins with high affinity to flour lipids. Cereal Chemistry 62, 284-289.

Zawistowska, U., Bietz, J.A., Bushuk, W., 1986. Characterization of low-molecular-weight protein with high affinity for flour lipid from two wheat classes. Cereal Chemistry 63, 414-419.

Zawistowska, U., Bushuk, W., 1986. Electrophoresis characterisation of low-molecular-weight wheat protein of variable solubility. Journal of the Science of Food and Agriculture 37, 409-417.

Zhen, Z., Mares, D., 1992. A simple extraction and one-step SDS-PAGE system for separating HMW and LMW glutenin subunits of wheat and high molecular weight proteins of rye. Journal of Cereal Science 15, 63-78.

Appendix

Copy of the report on analysis of amino acid content of sample provided by Australian Proteome Analysis Facility (APAF)

AMINO ACID ANALYSIS REPORT

Customer Details:

Thu Vu
Food Science Australia
671 Sneydes Road
Ph: 03 9731 3466
Fax: 03 9731 3250

02 October 2008

Sample Details:

Four samples were supplied for quantitative amino acid analysis

Analysis Details:

Performed by Prithi Lopez

- For Quantitative amino acid analysis samples underwent 24hr liquid hydrolysis in 6M HCl at 110°C. Cysteine and Tryptophan not analysed by this method.
- After hydrolysis all amino acids were analysed using the Waters AccQTag chemistry.
- Samples were analysed in duplicate and results are expressed as an average.

AMINO ACID ANALYSIS REPORT

Results for Quantitative Amino acid Analysis:

Sample Name : S1

Amino Acid	Amino Acid (-H_2O) * (mg/g of sample)	Amino Acid (free) ** (mg/g of sample)	Mole %
Aspartic acid + Asparagine	24.1	27.8	2.9
Serine	37.1	44.8	5.9
Glutamic acid + Glutamine	349.5	398.3	37.3
Glycine	21.4	28.2	5.2
Histidine	16.2	18.3	1.6
Arginine	26.9	30.0	2.4
Threonine	18.6	21.9	2.5
Alanine	17.5	21.9	3.4
Proline	108.4	128.5	15.4
Tyrosine	27.1	30.1	2.3
Valine	31.7	37.5	4.4
Methionine	11.7	13.3	1.2
Lysine	8.7	9.9	0.9
Isoleucine	32.1	37.2	3.9
Leucine	55.8	64.7	6.8
Phenylalanine	41.7	46.8	3.9
Total	828.7		100.0

Australian Proteome Analysis Facility Ltd
Level 4, Building F7B, Macquarie University, Sydney, NSW, 2109,
Australia
Ph: +61 2 9850 6201 Fax: +61 2 9850 6200
www.proteome.org.au apafinfo@proteome.org.au

AMINO ACID ANALYSIS REPORT

Sample Name : S2

Amino Acid	Amino Acid (-H_2O) * (mg/g of sample)	Amino Acid (free) ** (mg/g of sample)	Mole %
Aspartic acid + Asparagine	24.2	28.0	3.2
Serine	35.8	43.2	6.2
Glutamic acid + Glutamine	304.6	347.1	35.5
Glycine	28.3	37.3	7.5
Histidine	15.3	17.3	1.7
Arginine	28.7	32.0	2.8
Threonine	19.1	22.5	2.8
Alanine	17.8	22.3	3.8
Proline	88.6	105.0	13.7
Tyrosine	28.2	31.4	2.6
Valine	27.1	32.0	4.1
Methionine	10.3	11.7	1.2
Lysine	14.1	16.1	1.7
Isoleucine	25.8	29.9	3.4
Leucine	48.2	55.9	6.4
Phenylalanine	34.8	39.1	3.6
Total	750.7		100.0

Australian Proteome Analysis Facility Ltd
Level 4, Building F7B, Macquarie University, Sydney, NSW, 2109,
Australia
Ph: +61 2 9850 6201 Fax: +61 2 9850 6200
www.proteome.org.au apafinfo@proteome.org.au

AMINO ACID ANALYSIS REPORT

Sample Name : P1

Amino Acid	Amino Acid (-H_2O) * (mg/g of sample)	Amino Acid (free) ** (mg/g of sample)	Mole %
Aspartic acid + Asparagine	19.4	22.5	4.6
Serine	20.2	24.4	6.3
Glutamic acid + Glutamine	137.4	156.5	29.1
Glycine	16.9	22.2	8.1
Histidine	10.0	11.3	2.0
Arginine	22.3	24.9	3.9
Threonine	13.3	15.7	3.6
Alanine	13.4	16.8	5.1
Proline	39.8	47.1	11.2
Tyrosine	14.4	16.0	2.4
Valine	18.0	21.2	4.9
Methionine	6.7	7.6	1.4
Lysine	14.3	16.3	3.0
Isoleucine	15.6	18.1	3.8
Leucine	29.1	33.7	7.0
Phenylalanine	19.1	21.4	3.5
Total	409.8		100.0

Australian Proteome Analysis Facility Ltd
Level 4, Building F7B, Macquarie University, Sydney, NSW, 2109,
Australia
Ph: +61 2 9850 6201 Fax: +61 2 9850 6200
www.proteome.org.au apafinfo@proteome.org.au

AMINO ACID ANALYSIS REPORT

Sample Name : GC

Amino Acid	Amino Acid (-H_2O) * (mg/g of sample)	Amino Acid (free) ** (mg/g of sample)	Mole %
Aspartic acid + Asparagine	22.3	25.8	3.3
Serine	30.8	37.1	6.0
Glutamic acid + Glutamine	259.0	295.1	34.3
Glycine	20.3	26.7	6.1
Histidine	14.3	16.1	1.8
Arginine	26.5	29.6	2.9
Threonine	17.2	20.2	2.9
Alanine	16.4	20.6	3.9
Proline	80.3	95.2	14.1
Tyrosine	23.2	25.8	2.4
Valine	26.1	30.8	4.5
Methionine	10.6	12.0	1.4
Lysine	13.2	15.0	1.8
Isoleucine	25.0	29.0	3.8
Leucine	46.5	53.9	7.0
Phenylalanine	33.2	37.3	3.9
Total	664.8		100.0

* Calculation based on amino acid residue mass in protein (molecular weight minus H_2O).

** Calculation based on free amino acid molecular weight.

Australian Proteome Analysis Facility Ltd
Level 4, Building F7B, Macquarie University, Sydney, NSW, 2109,
Australia
Ph: +61 2 9850 6201 Fax: +61 2 9850 6200
www.proteome.org.au apafinfo@proteome.org.au